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VOLUME VII—VIII



1945

ACADEMIC PRESS INC. PUBLISHERS  
NEW YORK, N. Y.

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# **Accelerated Growth of Albino Mice Fed Diets Containing Extracts of Pancreas or Liver<sup>1</sup>**

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Received March 7, 1945

## **INTRODUCTION**

White and Sayers (5) have reported that albino rats grow more rapidly when they are fed a diet in which the protein is furnished by a pancreas preparation (P-1) (2) than when casein is used as the protein source. Over a 28 day period, the average daily weight gain for the animals fed the pancreas diet was 5.9 g. and for the animals fed the casein diet, 4.4 g. The ratio of weight gain per g. of food consumed was the same for both groups (0.55).

It seemed possible that some non-protein material in the P-1 might be responsible for these results. Accordingly, P-1 was subjected to dialysis, and the dialyzate evaporated to dryness under reduced pressure. White and Sayers<sup>2</sup> added the dry dialyzate from a known amount of P-1 to the same amount of casein, and used this supplemented casein in another experiment. Rats fed the diet containing the supplemented casein grew at an average rate of 5.4 g. per day. It was concluded tentatively that the accelerated growth rate and increased food intake of the rats fed P-1 resulted from the presence of a water-soluble, non-protein factor. The failure of the animals fed the supplemented casein to grow as rapidly as those fed P-1 could be accounted for by assuming that only a portion of the factor had been removed by the dialytic procedure. This was supported by the finding that rats fed the dialyzed P-1 grew at a rate of 5.5 g. per day.

<sup>1</sup> A preliminary report of this investigation has appeared (1).

<sup>2</sup> We are grateful to Dr. Abraham White for his kind permission to mention this unpublished material.

## EXPERIMENTAL DATA AND DISCUSSION

It was decided that confirmation of the results obtained by White and Sayers should be sought, using a different animal species. Accordingly, male weanling mice (Sharp & Dohme, Swiss-Webster strain)

TABLE I  
*Composition of Diets Employed in Growth Studies*

Diet	A	B	C	D	E	F	G	H	I
	g.	g.	g.	g.	g.	g.	g.	g.	g.
Casein (Labco)	20.0	—	21.4	—	20.0	20.0	20.0	20.0	20.0
P-1	—	20.0	—	22.0	—	—	—	—	—
Hydrogenated cotton seed oil (Primax)	30.0	30.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Corn oil	2.0	2.0	—	—	2.0	—	—	—	—
Sucrose	—	—	15.0	15.0	—	15.0	15.0	15.0	15.0
Corn starch	—	—	34.6	34.0	—	30.7	30.0	29.0	28.0
Glucose (Cerelose)	20.0	20.0	—	—	20.0	—	—	—	—
White dextrin	22.7	22.7	—	—	26.7	—	—	—	—
Cellu flour	2.0	2.0	—	—	2.0	—	—	—	—
Salt mixture <sup>1</sup>	2.0	2.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Yeast <sup>2</sup>	—	—	5.0	5.0	—	5.0	5.0	5.0	5.0
Liver extract <sup>3</sup>	1.0	1.0	—	—	—	—	1.0	—	—
Vitamin B mixture <sup>4</sup>	0.024	0.024	—	—	0.024	0.024	—	—	—
Choline chloride	0.2	0.2	—	—	0.2	0.2	—	—	—
Vitamin A, D, and E concentrate <sup>5</sup>	0.1	0.1	—	—	0.1	0.1	—	—	—
AI	—	—	—	—	—	—	—	2.0	—
AS	—	—	—	—	—	—	—	—	3.0
Biotin in $\mu\text{g}./\text{g}$ . (by <i>L. casei</i> E assay)	—	—	—	—	none	0.10	0.11	0.10	0.10
Folic acid in $\mu\text{g}$ . potency 40,000 per g. (by <i>L. casei</i> E assay)	—	—	—	—	0.38	1.2	1.8	1.0	1.0

<sup>1</sup> Hubbell, R. B., Mendel, L. B., and Wakeman. A. J., *J. Nutrition* **14**, 273 (1937).

<sup>2</sup> Anheuser Busch, Strain G.

<sup>3</sup> Wilson's 1:20 powder.

<sup>4</sup> Supplies 0.8 mg. thiamine hydrochloride, 1.6 mg. riboflavin, 0.8 mg. pyridoxin hydrochloride, 4.0 mg. niacin, 4.4 mg. calcium pantothenate, 4.0 mg. *p*-aminobenzoic acid, and 8.0 mg. inositol per 100 g. of diet.

<sup>5</sup> Supplies 1 mg. 2-methyl-1,4-naphthoquinone diacetate, 4 mg.  $\alpha$ -tocopherol, 900 U.S.P. units vitamin A, and 180 U.S.P. units vitamin D per 100 g. diet.

In addition, each animal receiving Diets C, D, G, H, and I was given 0.1 ml. codliver oil daily.

were fed Diets A and B (Table I). The growth and food consumption data are given in Table II. It is evident that the average daily weight gain was slightly higher for the mice fed casein than for the mice fed P-1. However, the diets employed by us differed in one important respect from the diets employed by White and Sayers. The latter workers used yeast and codliver oil as vitamin supplements; our diets

TABLE II  
*Growth Rates and Food Consumption of Mice on Various Diets*

Diet	No. of Mice	Av. Initial Wt. g.	Av. Final Wt. g.	Av. Daily Wt. Gain g.	Av. Daily Food Consumption g.	Av. N Content of Carcasses per cent
A—After 10 days						
A	10	7.4	12.9	0.55	1.74	—
B	10	7.2	12.6	0.54	1.84	—
C	10	7.2	10.6	0.34	1.40	—
D	10	7.3	12.4	0.51	1.70	—
E	10	7.7	10.9	0.32	—	—
F	10	7.6	10.7	0.31	—	—
G	10	7.6	13.0	0.54	—	—
H	9	7.5	12.3	0.48	—	—
I	10	7.6	13.2	0.56	—	—
B—After 20 days						
A	10	7.4	21.0	0.68	2.52	—
B	10	7.2	19.3	0.60	2.54	—
C	10	7.2	16.4	0.46	1.89	—
D	10	7.3	19.9	0.63	2.47	—
E	10	7.7	16.6	0.44	—	2.80
F	10	7.6	18.2	0.53	—	2.71
G	10	7.6	19.7	0.60	—	2.79
H	9	7.5	20.9	0.67	—	2.73
I	10	7.6	21.1	0.68	—	2.80

were supplemented with liver extract, the pure B complex factors, and a concentrate containing  $\alpha$ -tocopherol and vitamins A and D.

When Diets C and D (Table I) containing yeast and codliver oil as vitamin sources were fed, it was found that the animals fed casein grew at a slower rate than those fed P-1 (Table II). It was noted, however, that the growth rates of the animals fed the diets containing P-1 were essentially the same with either type of vitamin supplement. These data made it probable that an unknown factor assumed to be present in adequate amount in P-1 was also present in the vitamin

supplement used in Diets A and B. If so, this would explain the apparently accelerated growth of the animals fed Diet A.

In order to find out, if possible, whether the postulated factor was in the pure B vitamin mixture or in the liver extract, mice were fed Diets E, F, and G (Table I), containing as vitamin B supplements the pure B factors alone, yeast plus the pure B factors, and yeast plus liver extract, respectively. The results, listed in Table II, appear to indicate that the liver extract used was a source of the factor. Neither yeast alone nor the pure B vitamin mixture alone appeared to contain adequate amounts of it, although some increase in growth rate occurred when both yeast and the pure B vitamin mixtures were present in the same diet (Diet F).

Some preliminary attempts to concentrate the active material present in P-1 have been made. To the filtrate obtained after treatment of P-1 with 8 vol. of water acidulated to pH 4.8 with HCl were added two vol. of acetone. The precipitate (AI) was dried with acetone. The material soluble in the acetone-water solution (AS) was recovered by evaporating the filtrate to dryness under reduced pressure. Approximately 4-5 g. of AI and about the same amount of AS were obtained from 100 g. of P-1.

Diets H and I (Table II) containing, respectively, AI and AS, were fed to male weanling mice. Both diets were supplemented with yeast and codliver oil but no pure B vitamins were added. The amounts of AI and AS used were made relatively high in order to insure a response if the factor were present. The data obtained are given in Table II. It will be noted that the highest growth rates obtained in this study were obtained after feeding the two diets for 20 days. After 10 days, however, the rate of growth of the animals fed Diet H was appreciably less than that of the animals fed Diet I. In general, growth rates at 20 days are more reliable than those at 10 days, since mice invariably lose weight for a day or two after they are placed on a purified diet. In the average case, the initial body weight is regained in 3-4 days.

Woolley (6) has shown that inositol is essential for mice. Since our pure vitamin B mixture is lower in inositol than is recommended by Woolley and since pancreas contains an appreciable amount of inositol, it was thought that this might be the factor.

Two groups of mice were fed Diet C (Table I) and Diet C supplemented with 4 times the level of the pure B vitamins previously

employed by us. In addition to the pure B vitamin mixture already present, 200 mg. of inositol, 0.25 g. of choline chloride, 0.1 ml. of a biotin concentrate (50  $\mu$ g. per ml.) and 0.15 g. of a folic acid concentrate containing 500  $\mu$ g. per g. were added to each 100 g. of diet. The growth rates of mice fed these two diets were essentially the same, 0.57 and 0.59 g. per day, respectively, for a twenty day period.

As additional evidence that neither biotin nor folic acid can be the factor, diets causing marked differences in growth rates were found to contain essentially the same concentrations of these substances<sup>2</sup> (Tables I and II).

It should be pointed out that the gains in body weight reported in this paper represent true growth and not an abnormal fat deposition. This is indicated by the finding that the carcasses of the mice exhibiting rapid growth contain within experimental error the same percentage nitrogen as do the animals exhibiting slower rates of growth (Table II).

It was shown by Troescher-Elam and Evans (4) that the growth of male mice, fed a diet supplemented only with the pure B vitamins and codliver oil, could be improved by the addition of fresh beef liver or a concentrate prepared from fresh beef liver. They also found that increasing the casein and salt levels, or doubling the daily quota of pure B vitamins, or both, failed to improve the growth.

McIntire, *et al.* (3) have reported that liver extract is necessary for maximal growth of rats receiving the pure B vitamins. They suggest that the liver either furnishes a growth stimulating substance or furnishes a better medium for an intestinal flora that synthesizes a growth stimulating factor. This latter result was interpreted by Zucker and Zucker (7) as indicating that the factor was associated with proteins of good quality either as an impurity or as an essential amino acid, since they were able to achieve optimal growth in female rats by feeding a diet containing 27.9 per cent Labco casein ( $N \times 6.25 = 24$  per cent). They concluded that the effect of liver extract could be eliminated by increasing the level of casein.

To determine whether or not this conclusion applies to the growth of mice, a study was made using diets containing, respectively, 20 per cent casein and 35 per cent casein, separate diets with each casein

<sup>2</sup> We wish to thank Dr. L. D. Wright and Mrs. H. R. Skeggs, who determined the biotin and folic acid contents of these diets.

level being supplemented with 5 per cent yeast, 5 per cent yeast plus 1 per cent liver extract, the pure B vitamins, or the pure B vitamins plus 1 per cent liver extract. The remainder of each diet was the same as Diet A in Table I except that the liver extract and vitamin B mixture were omitted. The white dextrin content was adjusted so that all ingredients remained on a parts per 100 basis.

Eight male weanling mice were fed each of the eight diets. The growth results obtained are shown in Table III. It is evident that for

TABLE III  
*Effect of Liver Extract on the Growth of Mice Receiving Either  
20 Per Cent or 35 Per Cent Casein for 20 Days*

Vitamin Supplement	Weight Gains of Mice				
	20 Per Cent Casein	35 Per Cent Casein	Difference	<i>t</i> <sup>2</sup>	<i>P</i> <sup>3</sup>
Synthetic B vitamins	13.6±0.6 g. <sup>1</sup>	11.6±0.7 g.	2.0 g.	2.16	0.045
Synthetic B vitamins plus 1 per cent liver extract	15.1±0.5 g.	13.9±0.4 g.	1.2 g.	2.02	0.062
Dif.	1.5 g.	2.3 g.			
<i>t</i>	2.02	2.86			
<i>P</i>	0.002	0.012			
5 per cent yeast	12.4±0.7 g.	10.4±0.7 g.	2.0 g.	2.04	0.059
5 per cent yeast plus 1 per cent liver extract	14.2±0.4 g.	13.6±0.6 g.	0.6 g.	0.85	0.416
Dif.	1.8 g.	3.2 g.			
<i>t</i>	2.18	3.61			
<i>P</i>	0.046	0.003			

$$^1 \text{Std. error} = \sqrt{\frac{\sum d^2}{N(N - 1)}}.$$

$$^2 t = \frac{m_1 - m_2}{\sqrt{S.E._1^2 + S.E._2^2}}.$$

<sup>3</sup>*P* = probability that difference is not significant.

mice the liver extract not only exerts its effect with both 20 per cent and 35 per cent casein, but that the effect with 35 per cent casein is greater than with 20 per cent casein. It can also be noted that, in all pairs, growth was better with the lower percentage of casein. We cannot at present explain this observation, which, incidentally, has also been noticed when whole egg protein instead of casein was fed.

## SUMMARY

From liver extract and pancreas a factor can be obtained which has an accelerating effect on the growth rate of mice. This factor is not present in yeast nor is it one of the known B complex factors. With mice the effect can be demonstrated with diets containing 35 per cent as well as 20 per cent casein.

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# Biotin Deficiency in Relation to Reproduction and Lactation<sup>1</sup>

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Received January 17, 1945

## INTRODUCTION

Much of the recent work that has been reported on biotin deficiency in rats has been confined to the external effects produced in the young rat fed a raw egg-white-containing diet complete except for biotin (1, 2, 3). In connection with the cure of the syndrome produced by this deficiency it has been shown that biotin is also one of the growth vitamins. Nielsen and Elvehjem (4) have shown that 2 micrograms of biotin per day per rat will cause a small but definite gain in rats fed a ration which produces the "spectacled eye" condition. Later, they (5) showed that with the addition also of folic acid much better growth was obtained. However, succinylsulfathiazole had been administered with the diet.

We wish to report a brief study in which we have shown that as a growth vitamin the need for biotin begins early in intra-uterine life in the rat.<sup>3</sup>

## METHOD

The rats used in this investigation came from our stock colony from the same line of rats. The albinos had been inbred for 60 generations, and the black rats, a cross of an albino male of this line and a black rat, back-crossed for 20 generations, were

<sup>1</sup> Published with the approval of the Director as Paper No. 2212, Journal Series, Minnesota Agricultural Experiment Station.

<sup>2</sup> Died March 8, 1944.

<sup>3</sup> This study was undertaken to determine the cause of an unusual condition existing in some new-born pigs whose mothers had been fed large quantities of unused eggs from hatcheries. Many of the pigs were born dead or in a moribund condition; some were paralyzed and the few that were born alive survived only a few days, apparently starving to death.

sib-mated for 14 generations. This line was chosen because of very low resorption in reproduction. The estrus cycle was followed in all of the rats and as soon as the vaginal smear showed the epithelial stage of estrus the rats were mated to a normal male of the same line from our stock colony. All litters were reduced in number to 4 rats and weaned when 28 days old.

Virgin rats 90 to 100 days old and ranging in weight from 150 to 190 g. were separated into five groups. Groups I, II, III, and IV were fed the same basal ration consisting of powdered egg albumin<sup>4</sup> 30, lard 5, salt mixture 4, codliver oil 2, wheat embryo oil 0.8, choline chloride 0.1, and sucrose 58.1. Group V was fed an identical ration except that 15 per cent of highly purified casein replaced 15 per cent of the powdered egg albumin. The vitamin supplements were fed daily in glass caster cups at the following levels: thiamine hydrochloride 80 µg., riboflavin 160 µg., pyridoxin 120 µg., calcium pantothenate 100 µg., and biotin, when administered, 2 µg.

### RESULTS AND COMMENTS

Table I summarizes the reproduction record of all groups.

The behavior of the rats in Group I indicates that they had stored sufficient amounts of biotin from their previous ration to enable them to give birth to fairly large first litters but only three of the mother rats were able to rear their young to the weaning age. Of the 23 rats that were nursed only 12 survived to the 28th day. At weaning, these rats were very much undersized compared to weanling rats of this same line in our stock colony, the weights of which range from 45 to 50 g. On weaning, the young rats were fed the basal ration and all the supplements including 2 µg. of biotin. When these rats were 56 days old they were still greatly below normal weight and showed symptoms of biotin deficiency, as indicated by soft downy fur, a peculiar hopping gait and, in a few cases, a reddish brown pigmentation under the fur on the back. One of the young rats became badly paralyzed and died the fifth week after birth.

The three albino mothers were unable to rear their young to the weaning age. One litter was killed by the mother when 21 days old and two litters starved because of the inability of the mothers to lactate.

Three rats of this group were remated after weaning their first litters. The records of these matings are shown in Table I. In all of these rats, the estrus cycle and implantation were undisturbed by the biotin deficiency and gestation was normal at least until the 11th to 13th day as was shown by the appearance of the erythrocyte sign.

<sup>4</sup> This powder readily dispersed in water and coagulated on heating.

TABLE I  
*Summary of Birth Record*

Group	Rat	First Litter		Second Litter		Implantations		Resorptions		Average Food Intake Per Week
		No.	Avg. Wt. Birth g.	No.	Avg. Wt. Birth g.	First Litter	Second Litter	First Litter	Second Litter	
I Basal ration, no biotin	B1	10	4.6	22.5						67
	B2	10	5.2	28.6						67
	B3	7	4.4	25.3						60
	A4	4	5.0							66
	A5	10	4.4	2 (dead)	4	6	11	2	9	72
	A6	3	5.6				4		4	78
II Basal ration and 2 $\mu$ g. biotin	A7	12	4							73
		(2 dead)								
	A8	10	4.3							60
	A9	3	4.5							66
	A10	10	4.2							50
	A11	4	4							70
III Basal ration and 6 $\mu$ g. biotin	B12	6	5	45.5						70
	A13	4	5							58
	A14	10	4	36						96
	A15	6	4.6	25.5						68
	B16	4	5	35.5						74
	B17	4	7	25						79
IV Basal ration, no biotin	A18	(4 dead)						6	2	73
	A19							10	10	65
V Casein, egg white ration, and biotin	A20	9	4.4							67
	A21	10	4.4	34						67
	A22	8	3.7	33						65

Resorption began early in the gestation period of rats A5 and A6. The former rat had 9 complete resorptions and two still-born undeveloped feti; the latter showed complete resorption on the 21st day of gestation. Resorption was in progress on the 15th day of gestation in rat B2.

The mother rats of this group showed symptoms of biotin deficiency, such as denuded areas around the eyes, nose, and on the abdomen, erythematous areas on the face and head, brown pigmentation of the skin on the back, and a peculiar unsteady gait. The black rats did not show a depigmentation of the pelage as reported by Emerson and Keresztesy (1). This was also true of the young black rats raised on the deficient diet.

The control rats, Groups II and III, were fed the basal diet and all the supplements including biotin. All of the rats of Group II except rat B12 destroyed their litters within the first week after birth. This destruction of young was probably due to a deficiency of biotin in the ration. All of the rats of this group were albinos except B12 which was a black rat. The relationship of these rats has been previously explained. In Group I, the 3 black rats reared their young on a biotin-free ration while the albinos failed. This failure on the part of the albinos of both groups lends support to Nielsen and Elvehjem's (4) finding that albinos are more susceptible to a biotin deficiency than piebald rats. The young of rat B12 were weaned when 28 days old. The average weight was 49 g. which is close to the weaning weight of our stock rats of this line.

Because of the unsatisfactory behavior of Group II, Control group III was started using the same ration but increasing the daily allowance of biotin to 6  $\mu$ g. One albino in this group killed her young four days after birth. The remaining rats were able to rear their young. However, as seen from Table I, their weaning weight was about one-half of what it should be, showing that either biotin or some other factor, possibly folic acid, was deficient in the mothers' diet.

The behavior of the rats in Group IV is further support to the supposition that biotin is needed for reproduction. These rats had been fed the biotin-free ration for five weeks before being mated in order to deplete their store of biotin. Neither rat was able to produce viable young and there was complete resorption in the case of rat A18.

To determine if reduction of the egg albumin in the ration would improve the diet for reproduction and lactation, 4 rats (Group V)

were fed a ration identical with the basal ration except that 15 per cent of the egg albumin was replaced by 15 per cent of purified casein. The supplements included biotin. It is evident from the record of these rats, Table I, that this change in the diet did not improve the ration for either reproduction or lactation. One litter died 4 days after birth, two litters were very much undersized at weaning, and the pregnancy of the remaining rat resulted in complete resorption of the feti.

From this brief investigation it is shown that uncoagulated egg albumin can form 30 per cent of the diet of female rats without upsetting the normal estrus cycle and that pregnancy advances normally until the 11th to the 13th day as shown by the appearance of the erythrocyte sign. However, the number of resorptions is very high in comparison with the number that has been found to occur in this line when the mother rat is fed our stock diet. The total number of rats born was 148, of which thirteen were born dead. Implantations in the 8 rats that were examined numbered 87 and resorptions 70. The implantations in 60 litters of this line in our stock colony numbered 558 with one resorption per litter. Lactation in all groups was very poor as is evidenced by early death of young, destroying of litters by the mother rat after several days of life, and the extremely poor growth of the young that did survive.

#### SUMMARY

Biotin is one of the factors needed for successful gestation and the birth of viable young in the rat and is probably a necessary factor in lactation; however, as folic acid was not included in the ration the effect of biotin on lactation is not positively shown.

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# Decomposition Products of Fibrinogen and Fibrin

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Received January 20, 1945

## INTRODUCTION

In recent years a considerable number of fibrinogen products have been prepared by a variety of procedures, and their stabilities vary widely from one product to another. One which decomposes in solution has been studied in this work. We have found that the decomposition products of fibrinogen and fibrin consist of two main components which we call the  $\alpha$ - and  $\beta$ -fibrinogen and fibrin derivatives, respectively. Our work shows that the fibrinogen and fibrin derivatives are very similar and that they may be identical.

## MATERIALS AND METHODS

*Fibrinogen and Fibrin.* The fibrinogen was prepared by alcohol precipitation (1). At the slaughter house, 4.5 gallons of bovine blood were mixed with 1 liter of anticoagulant ( $1.85\% K_2C_2O_4 \cdot H_2O$  and  $0.5\% H_2C_2O_4 \cdot 2H_2O$ ). The plasma was obtained by centrifugation. The following day 8 liters of it were diluted with an equal volume of physiological saline solution and cooled to  $0^\circ C$ . A 21% solution of alcohol was added in the cold in the form of a fine spray until the alcohol concentration reached 7%. The precipitate was collected by light centrifugation at  $0^\circ C$ . and washed 3 times with 1.5 liters of cold 7% alcohol in saline. The precipitate was dissolved in 1.5 liters of saline and stored at  $-40^\circ C$ . The percentage of clottable protein varied from 70 to 98% with different preparations. The preparations used in this work were over 96% pure.

Decomposed fibrinogen was obtained by allowing the solution to stand at room temperature until none of it could be clotted with thrombin. This required approximately 6 days. Owing to the fact that this preparation was made from alcoholic solutions, bacterial contamination was minimal. Consequently, microorganisms had little or no influence on the decomposition. To confirm this, an aliquot portion of fibrinogen was sterilized by Berkefeld filtration. It decomposed in the same manner as the main lot. It should be mentioned that we find bovine fibrinogen, in plasma, stable for longer periods of time than this purified material. Presumably this is due to the fact that organic reagents such as alcohol, chloroform, etc., activate the fibrinolytic enzyme (2).

The decomposed fibrin was obtained by first clotting the fibrinogen with a small amount of purified thrombin and allowing the clot to stand at room temperature until virtually all the fibrin had gone into solution. The thrombin added a negligible amount of protein. A small amount of insoluble material sometimes remained, which was also the case with fibrinogen. This material was removed by centrifugation. The decomposed fibrinogen and fibrin were dried from the frozen state.

*Electrophoresis.* Electrophoretic observations were made with a Tiselius apparatus equipped with a Schlieren lens and a Svensson diaphragm. Approximately 1% solutions of the dried samples were dialyzed overnight against 2 liters of buffer. Borate, phosphate, and acetate buffers were used at high, medium, and low pH values, respectively. The mobility values were calculated from descending boundary data, as recommended by Longsworth and MacInnes (3).

*Nomenclature.* In the case of fibrinogen, the major or heat-coagulable component is called the  $\alpha$ -fibrinogen derivative. The non-heat-coagulable component with the lower isoelectric point is called the  $\beta$ -fibrinogen derivative. Analogously, the fibrin fractions are designated  $\alpha$ -fibrin derivative and  $\beta$ -fibrin derivative, respectively.

## EXPERIMENTAL

*Electrophoretic Mobility Curves.* Fig. 1 shows the electrophoretic data obtained in the studies of the decomposed fibrinogen and fibrin. The similarity in the curves of both these preparations is evident. Although a sizable fraction of the original protein goes into the  $\beta$ -derivative, the electrophoretic properties of the  $\alpha$ -derivative are essentially those of fibrinogen (Fig. 2). The isoelectric points of all three: *i.e.*, fibrinogen,  $\alpha$ -fibrinogen derivative, and  $\alpha$ -fibrin derivative, are near pH 5.5.

In the neutral pH range, the  $\beta$ -derivatives have mobility values between those of albumin and  $\alpha$ -globulin. However, the isoelectric point is much lower than for either, the value being near pH 4.2. The relative amounts of the proteins are indicated by the inserts in Fig. 1, which are careful reproductions of electrophoretic patterns obtained at pH 5.96.

Within experimental error, the electrophoretic properties of the two  $\alpha$ -derivatives and the two  $\beta$ -derivatives are the same. It will be necessary to employ additional techniques to establish whether they are identical.

*Isolation of the  $\alpha$ -Derivative.* At room temperature, a 1% solution of decomposed fibrinogen in saline was mixed with a saturated solution of  $(\text{NH}_4)_2\text{SO}_4$ . At 25% of saturation there was virtually no precipitate. At 35% of saturation a fraction was removed by centrifugation and examined electrophoretically. It consisted of the  $\alpha$ -component and a small amount of impurity.  $(\text{NH}_4)_2\text{SO}_4$  was then added to 50% of

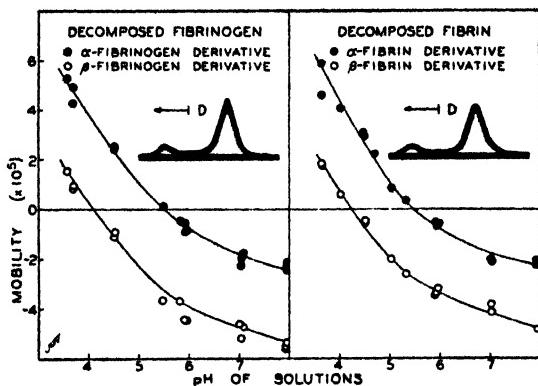


FIG. 1

Electrophoretic Mobility Curves of the Decomposition Products of Fibrinogen and Fibrin

The inserts are careful reproductions of the electrophoretic patterns at pH 5.96.

saturation. The precipitate was collected by centrifugation, dissolved in water, dialyzed until free of  $(\text{NH}_4)_2\text{SO}_4$ , and dried from the frozen state. The snow white protein was pure  $\alpha$ -fibrinogen derivative as judged from the electrophoretic pattern.

This  $(\text{NH}_4)_2\text{SO}_4$  fractionation was duplicated in every respect with decomposed fibrin.

*Partial Purification of  $\beta$ -Derivative.* A 1% solution of decomposed

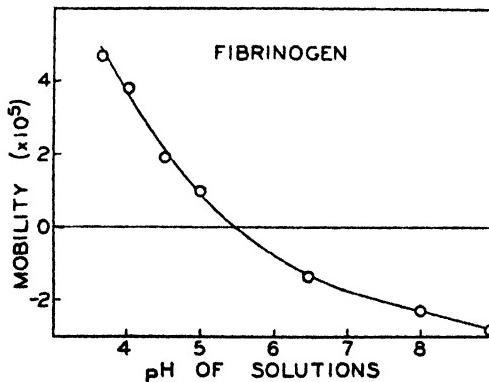


FIG. 2

The Electrophoretic Mobility Curve of Fibrinogen

fibrinogen was gradually warmed in a water bath. At 51°C., coagulation of the  $\alpha$ -fibrinogen derivative occurred. The temperature was then brought to 55°C. After 15 minutes, the heat-denatured protein was removed by centrifugation and the clear supernatant solution was dialyzed thoroughly. Approximately 0.1 N HCl was added to pH 4.3. The precipitate was collected by centrifugation, dissolved in water with the aid of alkali, and dried from the frozen state. Electrophoresis experiments showed that most of the protein was  $\beta$ -fibrinogen derivative, but there was also present a small amount of impurity.

The same procedures were followed with decomposed fibrin, and the results were in no way different from those with decomposed fibrinogen.

### DISCUSSION

The fact that we have often made fibrinogen preparations which do not decompose on standing leads us to assume that the products studied in this paper contained the fibrinolytic enzyme, and that we are dealing with the decomposition products which result from the action of that enzyme. It is quite possible that even more and smaller protein fragments are eventually produced through fibrinolysis, but it is evident that two of the first decomposition products are those which we have described. There is some evidence that these are not intermediate decomposition products in that we were unable to find any new changes even after many additional weeks of standing at room temperature.

It is interesting to recall that the fractions obtained by Garner and Tillett (4) are in many respects like those which we have described. Significantly, they used fibrinolysin from hemolytic streptococci to produce the effect. It is possible that the decomposition reactions are identical.

The isolation of the  $\alpha$ -derivative from both decomposed fibrinogen and fibrin offers new opportunities to study the nature of the alteration which thrombin produces in fibrinogen. If the  $\alpha$ -fibrinogen derivative and the  $\alpha$ -fibrin derivative are identical compounds which represent the greater portion of the original protein molecule, then obviously thrombin produces no alteration in the structure of the protein portion which becomes  $\alpha$ -fibrin derivative. We feel, therefore, that these proteins will furnish important opportunities for the study of fibrinogen and fibrin.

It is also possible to make new investigations in protein stoichiometry. Bergmann and Niemann (5) believe that the amino acids in bovine fibrin arrange themselves periodically along the length of the peptide chain. Each glutamic acid unit is separated from the previous and succeeding ones by seven other amino acids. This description of fibrin assumes that the periodic distribution holds throughout the length of the peptide chain. If this be true, analysis should show that  $\alpha$ -fibrin derivative has virtually the same amino acid distribution as the parent fibrin molecule. It is not impossible that the amino acid distribution is uneven in the fibrin molecule, and also in the original fibrinogen molecule.

### SUMMARY

Purified fibrinogen solutions which decomposed on standing contained two main protein components. One, which we call  $\alpha$ -fibrinogen derivative, has electrophoretic properties similar to those of fibrinogen. It is heat-coagulable at 51°C. It can be precipitated with  $(\text{NH}_4)_2\text{SO}_4$ , and has been isolated in pure form. The other component, which is present in smaller concentration, has been called  $\beta$ -fibrinogen derivative. Its isoelectric point is pH 4.2. It is not heat-coagulable, it is soluble in  $(\text{NH}_4)_2\text{SO}_4$ , and has been obtained in partially purified form.

When a sample of the fibrinogen was clotted with purified thrombin, decomposition of the fibrin took place in a manner analogous to the decomposition of fibrinogen. The fibrin clot lost its structure and went into solution. The decomposed material consisted of two chief components. Their properties are so similar to the properties of the decomposed fibrinogen products that the respective proteins may be identical.

We wish to thank Miss Grace Moyer for valuable technical assistance.

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# The Action of Synthetic Detergents on Protyrosinase \*

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Received January 22, 1945

## INTRODUCTION

Synthetic detergents are capable of producing a diversity of effects when added to various proteins. Among these reactions, that of precipitation of proteins by anionic detergents has recently been described by Putnam and Neurath (1). These authors noted particularly that horse serum albumin, when precipitated by sodium dodecyl sulfate, could be dissociated, by means of barium salts, into regenerated protein and insoluble barium dodecyl sulfate. Since protyrosinase from the egg of the grasshopper is readily activated and converted into tyrosinase by anionic detergents (2), it becomes of some interest to determine the effects of barium salts upon the protein detergent complex thus formed. The present communication deals primarily, in a qualitative way, with the effects of barium chloride upon the activity of tyrosinase activated by the anionic detergents, sodium dodecyl sulfate and di-octyl sodium sulfosuccinate (aerosol OT).

## MATERIALS AND METHODS

Protyrosinase was prepared from the egg of the grasshopper, *Melanoplus differentialis*, and standardized as previously described (3). Concentrations of detergents used were those already known to produce maximum activity of enzyme samples (4). Barium chloride solutions were added to detergents or protein detergent complexes in various concentrations but always keeping the molecular proportions of barium and detergent constant. Filtration of precipitates through double thickness of Whatman No. 42 filter paper as well as centrifugation were employed in obtaining samples for analysis. The presence of detergent was checked by additions of inactive enzyme, protyrosinase, to the sample as well as by the formation of precipitates upon the addition of barium chloride to the clear portions. Protyrosinase, activated

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\* Aided by a grant from the Rockefeller Foundation for research in cellular physiology.

by exposure to 70°C. was also used in checking effects of detergents on samples of enzyme activated by other than chemical reagents. All activity determinations were carried out in Warburg manometers and with technics as previously indicated (5). Activity values in all cases are expressed in terms of aerosol OT activated enzyme as 100%.

Considerable variation in absolute activity values was noted between different enzyme extracts and in the same extract with increasing age. However, constant qualitative differences between the filtrates were always obtained.

### RESULTS AND DISCUSSION OF EXPERIMENTS

Filtrates from protyrosinase extracts gave negative activity values. However, when substrate, tyramine hydrochloride, was added to the filter paper a marked darkening was produced indicating adsorption and activation of the enzyme on the surface of the paper. No active or inactive form of the enzyme, however, was found to pass through the filter paper as evidenced by the lack of activity and negative protein tests (Millon's and xanthoproteic) in the filtrate.

When  $\text{BaCl}_2$  (0.01%) was added to protyrosinase and the solution filtered, the filtrate gave negative reactions for both barium and enzyme, showing that barium undoubtedly combined with the enzyme protein. Active enzyme is also produced on the filter paper as in the previous experiments with protyrosinase alone, indicating that barium in no way interferes with this reaction of the enzyme protein.

Detergents, aerosol OT, and sodium dodecyl sulfate in concentrations employed, when filtered, were recovered in the filtrates unchanged (Table I).  $\text{BaCl}_2$  added to aerosol OT or sodium dodecyl sulfate in equimolecular proportions (1 NaDS:1  $\text{BaCl}_2$ ), (1 aerosol OT:0.5  $\text{BaCl}_2$ ), produced complete precipitation of the detergent and these precipitates did not pass through the filter paper (Table I). Protyrosinase samples, when mixed with these precipitates were partially activated, due probably to their adsorption and subsequent activation on the surface of the finely divided particles.

Aerosol OT when added to protyrosinase in a concentration of 0.01% gave maximum activation of the enzyme samples. When such a mixture is filtered no active enzyme or detergent can be demonstrated in the filtrate (Summary 1, b). Thus at this, the minimum concentration of aerosol OT for maximum activation of the enzyme, all detergent seems to be tied up or involved with the prosthetic group (?) of the enzyme protein complex. Tests of the filtrate for protein (Millon's and xanthoproteic) are also negative. Aerosol OT, as such,

**TABLE I**  
*Results of Filtration Experiments*

Composition of mixture	Aerosol—OT		NaDS	
	% activity of filtrate	Remarks	% activity of filtrate	Remarks
(E+X)	0	Activated enzyme does not pass through filter paper. OT goes through—filtrate negative for protein.	15.8	Some activated enzyme passes through filter paper. No NaDS goes through. Filtrate positive for protein.
(X)	0	OT not adsorbed on paper. Passes through unchanged.	0	NaDS not adsorbed on paper. Passes through unchanged.
(X+BaCl <sub>2</sub> )	0	OT pptd. by BaCl <sub>2</sub> . No OT in filtrate.	0	NaDS pptd. by BaCl <sub>2</sub> . No NaDS in filtrate.
(E+X+BaCl <sub>2</sub> )	0	No enzyme goes through filter paper. OT in filtrate.	14	Some active enzyme passes through filter paper. Filtrate negative for NaDS.
(E+X+BaCl <sub>2</sub> ) + add. E to filtrate	100	OT in filtrate. No active E goes through.	78.5	No NaDS in filtrate—activating protein? Compare with (E+Ba+X)+E.
(E+X+2 BaCl <sub>2</sub> )	0	No enzyme, or OT in filtrate.		
(E+X+2 BaCl <sub>2</sub> ) + add. E to filtrate	0	Activator removed. No OT in filtrate.		
(E+Ba+X)	0	No E in filtrate—OT in filtrate.	13.7	Same as (E+NaDS+Ba).
(E+Ba+X)+add. E to filtrate	100	OT in filtrate. No active enzyme goes through.	18	Ba changes reaction to added E [compare with (E+X+BaCl <sub>2</sub> )+E].

E = protyrosinase.

X = detergent—for aerosol OT = 0.1%—for NaDS = 0.4 mg.

BaCl<sub>2</sub>—equimolecular concentrations used unless specified to the contrary.

% activity in terms of aerosol OT activated controls.

can be recovered in the filtrate in increasing amounts when concentrations greater than 0.01% are used (Summary I, c). Detergent is apparently held by (adsorbed?) the protein complex in definite amounts and only when it is present in excess of these does it appear in the filtrate. No protein is found in the filtrate even at the higher concentrations of detergent.

If protyrosinase were to behave like blood serum albumin, as demonstrated by Putnam and Neurath (1), one might reasonably expect the addition of  $\text{BaCl}_2$  in equimolecular proportions to aerosol OT, to break up the protein detergent complex into a protein and an insoluble barium compound. A point of further interest is whether

*(I) Summary of Reactions with Acrosol OT and Barium*

Reaction Mixture	Results	Remarks
a) $\text{Ba} [0.5x^*] + \text{OT} [x]$	$\rightarrow$ insol pptd BaOT	All OT precipitated as Ba salt
b) Protyrosinase + OT [0.01%]	$\rightarrow$ tyrosinase OT	All OT involved with prosthetic group? None in filtrate when filtered
c) Protyrosinase + OT [0.10%]	$\rightarrow$ tyrosinase OT	One part OT with prosthetic group, nine parts with carrier protein. OT in filtrate
d) Tyrosinase OT [0.1%] + Ba; filtrate + protyrosinase $\rightarrow$ 100% activity		Excess OT on carrier protein supplanted by Ba making OT available in filtrate.
e) Tyrosinase OT [0.01%] + Ba; filtrate + protyrosinase $\rightarrow$ O activity		No OT available for filtrate from carrier protein; Ba unable to dislodge OT from prosthetic group.
f) Protyrosinase + Ba $\rightarrow$ protyrosinase Ba		O activity
g) Protyrosinase + Ba + OT [0.01%] $\rightarrow$ tyrosinase		100% activity No effect of Ba.

Note: 0.01% acrosol OT = minimum concentration for maximal activation.

\* Equimolecular ratio of  $\text{BaCl}_2$  to aerosol OT (OT:BA::1.0:0.5).

## (II) Summary of Reactions with NaDS and Barium

Reaction Mixture	Results	Remarks
a) Ba [x]* + NaDS x	insol. pptd. BaDS	All NaDS precipitated.
b) Protyrosinase    NaDS [0.2 mg.] → tyrosinase		Active enzyme passes through filter (filtrate 7.4% active).
c) Protyrosinase + NaDS [0.4 mg.] → tyrosinase DS		Active enzyme passes through filter (filtrate 15.8% active).
d) Tyrosinase DS [0.2 mg.] + Ba; filtrate + E → 0 activity		Filtrate negative for NaDS.
e) Tyrosinase DS [0.4 mg.] + Ba; filtrate + E → 78.5% act.		Filtrate negative for NaDS. Activity due to protein.
f) Tyrosinase DS [0.2 mg.]; filtrate + E → 14.5% act.		
g) Tyrosinase DS [0.4 mg.]; filtrate + E → 118 % act.		

Note: 0.4 mg NaDS = 33% excess of maximal activation amounts.

\* Equimolecular ratio of BaCl<sub>2</sub> to NaDS (Ba : NaDS :: 1.0 : 1.0).

the protein thus recovered would still be in the active state or would revert to the protyrosinase condition.

Additions of BaCl<sub>2</sub> to protyrosinase and aerosol OT (0.01%) (Mol. ratio aerosol OT:Ba = 1:0.5), gave no active enzyme, protein or aerosol OT in the filtrate (Summary I, e). However, when BaCl<sub>2</sub> and aerosol OT of 0.10% are employed, aerosol OT but no active enzyme or protein was present in the filtrate (Table I, Summary I, d). When protyrosinase is added to these filtrates the activity increased from zero with 0.01% aerosol OT to 100% with 0.10% (Table I, Summary I, e, d). With the lower concentrations of detergent no aerosol OT is available for the filtrate from the carrier protein since the barium is unable to dislodge the aerosol OT from the prosthetic group. For the higher concentrations of activator, on the other hand, the excess aerosol OT on the carrier protein is apparently supplanted by the barium, thus making aerosol OT available in the filtrate as shown by its high activity (100%) upon the addition of protyrosinase. There appears to be competition between the enzyme surface and the

aerosol OT for added barium. Doubling the proportion of BaCl<sub>2</sub> and using 0.10% aerosol OT gave negative aerosol OT and protein reactions in the filtrate (Table I). Obviously, in this latter case all the aerosol OT was precipitated out by the excess barium added. Protein tests (Millon's and xanthoproteic) gave negative results when applied to all filtrates from aerosol OT and barium treated enzyme. Aerosol OT apparently does not readily cause fragmentation or splitting of the enzyme protein complex in such a way as to allow active enzyme or other protein to pass through a No. 42 Whatman filter paper.

Protyrosinase, activated with sodium dodecyl sulfate is of especial interest when compared to that activated by aerosol OT. The activation reactions although probably similar in nature are accompanied by quite different results for the remainder of the protein complex. Filtrates from sodium dodecyl sulfate treated protyrosinase (within concentration limits) are negative for free sodium dodecyl sulfate but give positive protein reactions (Millon's and xanthoproteic) and also show the presence of active enzyme (Table I; Summary II, b, c; Fig. 1, C). The concentrations of sodium dodecyl sulfate referred to are those which always gave 100% activation of the protyrosinase. Apparently the enzyme protein in addition to being completely activated is sufficiently broken down or acted upon by the detergent to allow some active enzyme to pass through the filter. The activity of the filtrate due to this active enzyme increases with increased detergent concentration up to a maximum (1.2 mg.) at which value it tends to remain constant (Fig. 1, C). Since the amount of protyrosinase used is constant it seems reasonable to assume that once the concentration of sodium dodecyl sulfate which will completely break down or act upon the enzyme protein is reached, further addition of detergent can produce no more active enzyme in the filtrate (Fig. 1, C). Addition of protyrosinase to the filtrates, however, produced activity well above the aerosol OT control (Fig. 1, A).

These results seem of interest inasmuch as they indicate a marked difference in behavior from those obtained with aerosol OT. In the latter case excess aerosol OT was present and could be demonstrated in the filtrates while with sodium dodecyl sulfate no free detergent but active enzyme was found which upon addition of protyrosinase produced marked activity. Several possible interpretations suggest themselves and among them the following seem pertinent. *First*, sodium dodecyl sulfate in its activating action upon protyrosinase

produces a smaller active portion (prosthetic group?) along with a small carrier protein fraction. Parts of both fractions, as produced, are small enough in size to pass through a No. 42 Whatman filter. Addition of protyrosinase to such a filtrate produces marked activity

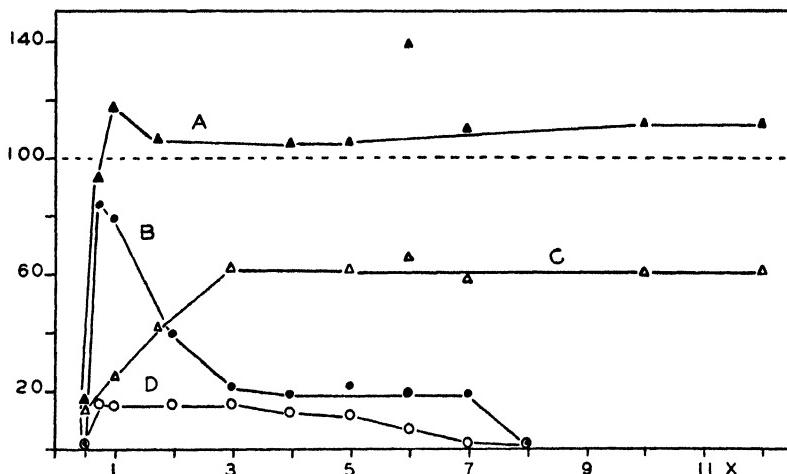


FIG. 1

Effect of Adding Protyrosinase to Filtrate of Sodium Dodecyl Sulfate Protein Complex

Ordinate, percentage activity in terms of aerosol OT control as 100%. Abscissa, concentration of reagents in terms of  $X = 0.4323 \text{ gg.} = 0.3 \text{ cc.}$

$4.99 \times 10^{-3} M$  NaDS (amount for maximum

activation). Molecular ratio of

NaDS to  $\text{BaCl}_2$ , 1:1.

A = (Protyrosinase + NaDS) filtered + protyrosinase to filtrate.

B = (Protyrosinase + NaDS +  $\text{BaCl}_2$ ) filtered + protyrosinase to filtrate.

C = (Protyrosinase + NaDS) filtered.

D = (Protyrosinase + NaDS +  $\text{BaCl}_2$ ) filtered.

Points represent averages from experiments, all with the same protyrosinase preparation.

due possibly to some autocatalytic action of the carrier protein or other similar factors present. Secondly, sodium dodecyl sulfate may combine with the protyrosinase in different degrees depending on the relative concentrations used. The enzyme protein dodecyl sulfate complex thus formed is not only active but also slightly soluble and

thus some passes through the filter. Increasing the sodium dodecyl sulfate concentration causes more dodecyl sulfate to be attached to the enzyme rendering it more soluble and hence more passes through the filter. (Note: Activity of the filtrate in fact never reaches 100% even with high concentrations of detergent.) Adding protyrosinase to this soluble less active enzyme dodecyl sulfate complex in the filtrate permits distribution of the sodium dodecyl sulfate over a larger amount of activated enzyme and thus increases the activity of the added protyrosinase to over 100%. If this interpretation were correct then the addition of barium to the sodium dodecyl sulfate protyrosinase combination should tend to make the enzyme dodecyl sulfate complex less soluble and hence less would pass through the filter. Additional data presented below add further possibilities to a clearer interpretation of the above suggestions.

Additions of  $\text{BaCl}_2$  to sodium dodecyl sulfate protyrosinase combinations at concentrations of 0.32 mg. to 2.1 mg. of detergent (5 times maximum activation concentration) allowed much smaller amounts of active enzyme to pass through to the filtrate than with detergent alone (Table I, Fig. 1, D). This decreased amount of active enzyme in the filtrate as above indicated, might be due to the fact that barium displaces or precipitates out the sodium dodecyl sulfate which otherwise is free to act upon the enzyme protein complex. No sodium dodecyl sulfate as such was present in the filtrate with barium treated samples. Positive tests for protein (Millon's and xanthoproteic) as well as for active enzyme are also given by the filtrates (Fig. 1, D). Protyrosinase added to the filtrate produced marked increase in its activity (Fig. 1, B). An explanation of this increased activity seems possible upon the assumption that activating substances are present in the filtrate and, as  $\text{BaCl}_2$ , used in equimolecular concentration with sodium dodecyl sulfate, would precipitate out all detergent, some factors other than activation by detergent seem present. As the concentrations of  $\text{BaCl}_2$  and sodium dodecyl sulfate are increased this filtrate activity due to the added protyrosinase is reduced and finally disappears, probably as the result of the effects of the increased concentrations of the reagents (Fig. 1, B). Since no free sodium dodecyl sulfate appears to be present in the filtrate, this marked activity occurring upon the addition of protyrosinase becomes of interest. Activating groups on the proteins in the filtrate may possibly account for the autocatalytic type of action on added protyrosinase.

In order to further test the effects of sodium dodecyl sulfate on the enzyme tyrosinase, samples of enzyme were activated by exposure to 70°C. (6). Tyrosinase, thus activated, has previously been shown to possess physical properties different from those of aerosol OT or sodium dodecyl sulfate activated enzyme and should afford rather a favorable enzyme protein complex upon which to test the effects of sodium dodecyl sulfate.

Protyrosinase added to heat activated enzyme produces no significant increase in activity (Table II, a, b). Filtrates from heat activated samples, with or without added protyrosinase, show no activity and give negative protein reactions. Apparently, no active prosthetic or other protein groups go through the filter under these conditions. Addition of BaCl<sub>2</sub> (within limits) does not change the activity of heat activated samples either with or without filtration (Table II, c, d). Heat activated tyrosinase, treated with sodium dodecyl sulfate, also shows no effect of the detergent upon either the filtered or unfiltered portion (Table II, e). However, upon the addition of protyrosinase both filtered and nonfiltered samples show marked activity due apparently to the free sodium dodecyl sulfate present (Table II, f). BaCl<sub>2</sub> added to sodium dodecyl sulfate treated heat-activated tyrosinase produces no significant differences in results over those obtained with sodium dodecyl sulfate alone (Table II, g). Addition of protyrosinase, however, produces activity in the filtrate but lower than that without the barium (Table II, h). Activity in the nonfiltered sample is increased to almost the same degree as with sodium dodecyl sulfate alone (Table II, g).

Since filtrates from sodium dodecyl sulfate treated samples of heat activated tyrosinase show no activity, passage of active prosthetic groups through the filter seems quite unlikely. Protein tests (Millon's and xanthoproteic) do, however, show the presence of protein in these filtrates and suggest that inactive protein may be split off in the treatment or that soluble non-active fractions are formed and these in turn pass through the filter. Inasmuch as BaCl<sub>2</sub> added to the sodium dodecyl sulfate and heat activated enzyme precipitates out the dodecyl sulfate as an insoluble barium salt which, in turn, does not pass through the filter, one is led to suggest the presence of carrier protein activating units in the filtrate.

The order of addition of either BaCl<sub>2</sub> or detergent to protyrosinase is without effect upon the activity of the mixture for either aerosol OT

TABLE II  
*Results of Experiments with Heat Activated Enzyme*

Composition of mixture, etc.	$\frac{70^{\circ}\text{C.}}{5^{\circ}}$ % activity	$\frac{70^{\circ}\text{C.}}{10^{\circ}}$ % activity	Remarks
a { (E+Δ) filtered (E+Δ) no filtration	0	0	No active enzyme or protein goes through filter. Activation due to heat.
	93	81	
b { (E+Δ) filtered+E (E+Δ) no filter+E	0	0	No activating substances in filtrate.
	98	84.2	No effect of added E.
c { (E+Δ)+Ba - filtered (E+Δ)+Ba - no filter	0	0	No active enzyme or protein goes through filter.
	90.9	87.6	No marked effect of Ba.
d { (E+Δ)+Ba - filtered+E (E+Δ)+Ba - no filter+E	0	0	No activating substances go through.
	92.3	86.5	No effect of added E.
e { (E+Δ)+NaDS no filter (E+Δ)+NaDS filtered	93	80	No effect of NaDS on activity.
	0	0	Negative for active enzymes, protein +, NaDS +.
f { (E+Δ)+NaDS no filter+E (E+Δ)+NaDS filtered+E	148	137	Added E activated by NaDS.
	97.6	93	Activating substances go through?
g { (E+Δ)+NaDS+Ba no filter (E+Δ)+NaDS+Ba filtered	93.3	83.2	No effect of Ba or NaDS.
	0	0	No active enzyme goes through (protein +).
h { (E+Δ)+NaDS+Ba no filter+E (E+Δ)+NaDS+Ba filtered+E	139	121.5	Additional E activated by BaDS? (or protein).
	62.2	57	Additional E activated by carrier protein?

E = protyrosinase.

Δ =  $70^{\circ}\text{C.}$

Concentration of reagents = minimum producing maximum activation of protyrosinase.

% activity in terms of aerosol OT activated controls.

or sodium dodecyl sulfate (Table I). When  $\text{BaCl}_2$  is first added to protyrosinase and then sodium dodecyl sulfate, the filtrate from such a mixture, when protyrosinase is added to it, shows a much reduced activity over that when the order of addition is reversed (Table I). In the case of aerosol OT, the activity of the filtrate upon the addition of protyrosinase is unchanged.  $\text{BaCl}_2$  apparently acts upon the protyrosinase in such a way as to reduce the effectiveness of the action of sodium dodecyl sulfate upon the protein molecule especially as regards the amount of activating fraction which passes through the filter. Since the filtrate activity is similar for enzyme dodecyl sulfate samples whatever the order of addition of barium or sodium dodecyl sulfate to the protyrosinase and if equal activity indicates the same amount of activating reagents present, then one would expect the addition of protyrosinase in the above cases to produce somewhat similar results. As a matter of fact, when  $\text{BaCl}_2$  is first added to protyrosinase and then sodium dodecyl sulfate there is very little increase in the filtrate activity upon the addition of protyrosinase. However, when sodium dodecyl sulfate is first added to protyrosinase and then  $\text{BaCl}_2$ , a marked increase in filtrate activity results upon the addition of protyrosinase. The barium protyrosinase complex apparently reacts to the sodium dodecyl sulfate in a manner quite different from that when barium reacts with the enzyme dodecyl sulfate combinations.

From the above results the authors are inclined to favor the first of the possible interpretations offered, *viz.*, that sodium dodecyl sulfate acts upon the enzyme protein complex in such a way as to produce its breaking down or fragmentation into smaller units which in turn pass through the filter and probably serve as activating centers for added protyrosinase.

#### CONCLUSION

Additional evidence that the protein carrier part of the tyrosinase molecule can be modified by treatment with various types of activators seems at hand. Similar conclusions were indicated in previous work on the effects of high temperatures (7). No evidence has been forthcoming to indicate a reversibility upon the addition of barium salts in the activation of protyrosinase produced by aerosol OT and sodium dodecyl sulfate. Effects of activating agents, *e.g.* sodium dodecyl sulfate, are apparently not confined to the prosthetic groups as such since they also cause fragmentation of a variety of complexes in

various ways (8, 9, 10, 11). In the case of sodium dodecyl sulfate for the present experiments, fragmentation of the complex apparently allows active enzyme, as well as other protein fractions, to pass through a No. 42 Whatman filter paper. With aerosol OT and heat, on the other hand, no active enzyme can be found in the filtrates. Further quantitative studies are necessary to determine the nature of the changes induced in the carrier part of the enzyme protein molecules by synthetic detergents.

#### SUMMARY

1. The anionic detergents, sodium dodecyl sulfate and dioctyl sodium sulfosuccinate (aerosol OT) activate protyrosinase with the production of tyrosinase. No marked differences in the catalytic properties of the enzyme so produced are evident.
2. Protyrosinase does not pass through a No. 42 Whatman filter paper. Activation takes place through adsorption on the filter paper.
3. BaCl<sub>2</sub> precipitates aerosol OT and sodium dodecyl sulfate from solution. When added to protein detergent complexes no reversibility in the activation of the enzyme occurs.
4. Evidence is presented suggesting a fragmentation of the enzyme protein complex by sodium dodecyl sulfate. The protein fragments thus formed pass through a No. 42 Whatman filter paper. Carrier protein fractions produced in this fashion may act as activators of protyrosinase.

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# The Sulfur Content of the Pepsin Digestible Fraction of Protein Concentrates

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Received December 13, 1944

## INTRODUCTION

The sulfur-containing amino acids and their role in nutrition have been extensively studied since Osborne and Mendel (1) showed cystine to be essential for the rat and Muldoon, Shipley, and Sherwin (2) demonstrated that inorganic sulfur could not be utilized by the dog. Sulfur, to be utilized in body proteins, must be supplied in the diet in the form of the amino acids cystine and methionine according to recent information. Since the requirements for these amino acids are high, some protein concentrates may be deficient in them. Such deficiencies may limit the nutritive values of these protein concentrates when used in practical diets.

The organic sulfur of most proteins appears to be in the form of methionine and cystine (or cysteine) (3). All of this sulfur is not necessarily available to the animal. Keratins have been shown to be indigestible except when specially treated (4). Any keratin, or similar protein present in a protein concentrate, is not acted on by the digestive enzymes to liberate the constituent amino acids and, as a result, is not available for the animal. Evans, Carver, and Draper (5) found the protein of a sample of finely ground feathers to be 81.6% indigestible with pepsin.

In experiments under way in this laboratory it became desirable to determine how much of the sulfur of protein concentrates is available for animal nutrition. The method used in this study was based on the assumption that all protein made soluble with pepsin is utilized by the animal.

## PROCEDURE

The "digestible" organic sulfur was determined on the samples of casein, herring fish meal, pilchard fish meal, dogfish meal, meat scrap, and feathers described by Evans, Carver, and Draper (5), and on the samples of soybean oil meal, cottonseed meal, and pear described by Draper and Evans (6). Determinations were also made on samples of other feeds.

Nitrogen was determined by the Kjeldahl-Gunning-Arnold method (7). Protein was calculated by multiplying nitrogen by 6.25.

Total sulfur was determined by the Parr bomb method (8) and the nitric-perchloric acid digestion method (9).

Inorganic and organic sulfur were determined as described by Evans and Greaves (10).

Indigestible protein was determined by the method of Almquist, Stockstad, and Halbrook (11).

The sulfur of the fraction of the protein concentrate not digested by pepsin ("indigestible" sulfur) was determined as follows: Two gram samples were extracted with ether in a Soxhlet extractor and digested with pepsin as described by Almquist, Stockstad, and Halbrook (11) for determining the indigestible protein fraction. The residue from the digestion was transferred to a 500 ml. Kjeldahl flask and sulfur determined by the nitric-perchloric acid digestion procedure (9).

TABLE I

*Pepsin Digestible and Indigestible Sulfur in Animal Protein Concentrates †*

Sample	Protein concentrate*	Protein	Total S	Inorg S	Indig S	Dig org S	Indig. protein	Indig S in indig protein <sup>9</sup>	Dig org S in total protein <sup>10</sup>	Gain per unit of protein	Protein quality index*
No.		per cent	per cent	per cent	per cent	g *					
542	Pilchard fish	71.6	0.88	0.07	0.10	0.71	5.8	1.7	0.99	8.4	83.5
703	Herring fish	73.6	0.93	0.10	0.12	0.71	6.5	1.9	0.97	8.3	80.5
720	Pilchard	60.0	0.71	0.06	0.10	0.55	5.0	2.1	0.92	7.9	83.5
719	Pilchard	62.0	0.74	0.06	0.11	0.57	5.2	2.1	0.93	7.8	83.0
722	Dogfish	73.2	0.78	0.13	0.14	0.51	3.5	4.1	0.70	7.1	70.6
723	Dogfish	69.2	0.75	0.13	0.15	0.47	3.6	4.3	0.68	7.1	71.2
721	Dogfish	70.8	0.93	0.29	0.20	0.44	5.0	4.0	0.62	6.8	77.1
724	Dogfish	70.2	0.78	0.17	0.16	0.45	3.7	4.2	0.65	6.5	70.2
725	Dogfish	82.1	0.76	0.14	0.13	0.49	3.1	4.1	0.60	5.3	44.5
579	Meat scrap	57.5	0.36	0.05	0.08	0.23	4.6	1.8	0.40	2.6	47.5
760	Feathers	81.8	2.05	0.05	1.77	0.23	67.8	2.6	0.29	0.8	14.9

† All percentages reported are based on the weight of total concentrate except that those in Column 9 are based on weight of indigestible protein and those in Column 10 are based on weight of total protein.

\* Evans, Carver, and Draper (5).

The "digestible" organic sulfur was calculated by subtracting the "indigestible" sulfur from the total organic sulfur.

### RESULTS

The total sulfur, inorganic sulfur, "indigestible" sulfur, and "digestible" organic sulfur for eleven animal protein concentrates are given in Table I. A comparison of the indigestible protein and the sulfur in this fraction is also given. The indigestible protein of dogfish meals contained the most sulfur (over 4.0%). This is even higher than feathers. The other fish meals had an indigestible protein fraction that contained less sulfur than feathers. While the indigestible protein of feathers contained almost the same percentage of sulfur as did the total protein, the indigestible protein of the other animal concentrates contained a much higher percentage of sulfur than did the total protein.

The same measurements are presented in Table II for ten samples of soybean oil meal, two samples of cottonseed meal, and two samples

TABLE II  
*Pepsin Digestible and Indigestible Sulfur in Plant Protein Concentrates†*

Sample	Protein concentrate	Protein	Total S	Inorg S	Indig S	Dig org S	Indig protein	Indig S in indig protein <sup>9</sup>	Dig org S in total protein <sup>10</sup>	Gain per unit protein
No.		per cent	per cent	per cent	per cent	g *				
565	Soybean oil meal	46.3	0.43	0.04	0.03	0.36	2.8	1.0	0.78	6.6
635	Soybean oil meal	48.6	0.45	0.04	0.06	0.35	2.8	2.2	0.72	6.4
958	Soybean oil meal	46.8	0.43	0.04	0.05	0.34	2.4	2.1	0.73	6.4
763	Soybean oil meal	46.8	0.44	0.04	0.06	0.34	2.7	2.2	0.72	6.2
706	Soybean oil meal	47.1	0.44	0.04	0.04	0.36	2.6	1.4	0.76	5.8
667	Soybean oil meal	46.8	0.45	0.04	0.05	0.36	2.7	1.8	0.76	5.7
759	Soybean oil meal	50.0	0.44	0.04	0.03	0.37	3.3	0.9	0.74	4.9
764	Soybean oil meal	46.1	0.41	0.04	0.04	0.33	2.3	1.4	0.72	4.9
704	Soybean oil meal	45.3	0.45	0.05	0.04	0.36	2.4	1.7	0.79	4.6
765	Soybean oil meal	46.6	0.42	0.04	0.04	0.34	2.2	1.6	0.73	3.5
761	Cottonseed meal	37.1	0.42	0.06	0.11	0.25	6.4	1.8	0.67	2.8
702	Cottonseed meal	45.0	0.49	0.01	0.13	0.35	8.5	1.6	0.77	1.2
755	Alaska peas	23.4	0.15	0.00	0.01	0.14	0.9	1.3	0.60	4.7
754	1st & best peas	27.7	0.14	0.01	0.01	0.12	1.0	1.1	0.42	4.5

† See footnote, Table I.

\* Draper and Evans (6).

of ground peas. The sulfur content of the indigestible protein varied from 0.9 to 2.2%. There appeared to be no relationship between this value and the method of processing the meal, or its nutritive value.

A coefficient of correlation of +0.925 was obtained between the "digestible" organic sulfur content of all the animal protein concentrates investigated and the gain in weight per unit of supplementary protein, using weight data reported by Evans, Carver, and Draper (5). A coefficient of correlation between "digestible" organic sulfur and

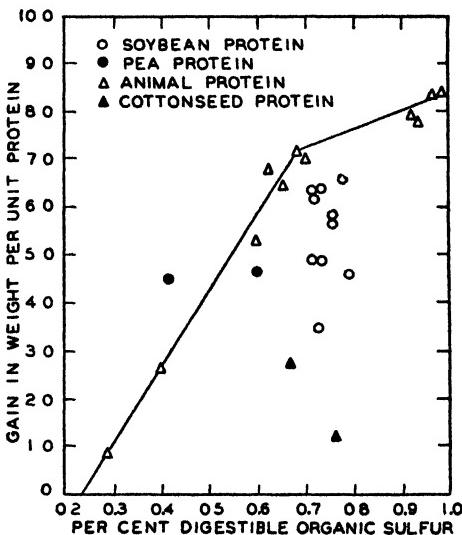


FIG. 1

Comparison of the Per Cent "Digestible" Organic Sulfur in the Total Protein of the Concentrate with the Gain in Weight of Chicks per Gram of the Concentrate Protein Consumed

protein quality index of +0.877 was obtained for the animal protein concentrates.

The "digestible" organic sulfur in the protein is plotted in Fig. 1 against the gain per unit of supplementary protein for chicks fed these concentrates (5, 6). There appears to be a relationship for the animal protein concentrates between the "digestible" organic sulfur and the gain per unit of supplementary protein. At a value of 0.68% "digestible" organic sulfur, the curve levels off, indicating that higher levels

of "digestible" organic sulfur may not be necessary or that other deficiencies also occurred above that level.

The values for soybean oil meal and cottonseed meal are on the right of the curve in Fig. 1, showing that growth was not as great as it should have been for the level of "digestible" organic sulfur present. While the "digestible" organic sulfur content may have been the limit-

TABLE III  
"Digestible" Organic Sulfur Content of Some Typical Feedstuff

Feedstuff	Samples	Digestible organic sulfur in feed			Digestible organic sulfur in protein		
		Range		Average	Range		Average
		number	per cent	per cent	per cent	per cent	per cent
Herring fish meal	2	0.75	0.71	0.73	1.05	0.97	1.00
Pilchard fish meal	3	0.71	0.55	0.61	1.00	0.92	0.95
Dogfish meal	5	0.51	0.44	0.47	0.69	0.60	0.65
Meat scrap	2	0.26	0.23	0.25	0.47	0.40	0.44
Casein	2	0.62	0.60	0.61	0.71	0.70	0.71
Whey	1			0.15			1.21
Feathers	1			0.23			0.29
Soybean oil meal	11	0.37	0.30	0.35	0.78	0.69	0.75
Cottonseed meal	2	0.35	0.25	0.30	0.77	0.67	0.72
Peas	5	0.14	0.09	0.12	0.60	0.42	0.49
Corn	1			0.10			1.01
Oats	1			0.11			1.05
Wheat	1			0.12			1.13
Millrun	1			0.16			1.08
Barley	1			0.10			1.08
Alfalfa	1			0.11			0.57
Cereal basal	1			0.13			1.09

ing factor for the peas, it was not the limiting factor for the soybean oil meals or cottonseed meals under the conditions of this experiment.

The "digestible" organic sulfur contents of a number of different feeds were determined and are presented in Table III.

#### DISCUSSION

A definite difference in nutritive values of different soybean oil meals was previously observed in this laboratory (6). Since the sulfur amino acids are the only amino acids that have been shown to be

deficient in soybean oil meal (12) these meals were analyzed for organic sulfur. This did not explain the differences in nutritive value. Since heating influences the availability of the organic sulfur (12) it appeared possible that the digestibility also might be influenced by heating. The method for "indigestible" sulfur was developed to determine if this is true. The *in vitro* digestion with pepsin and hydrochloric acid has been used successfully by Almquist and associates (11) for evaluating the digestibility of proteins. They presented evidence from the literature to show that this method gives results comparable to digestion trials with animals.

Under the conditions of this experiment the low "digestible" organic sulfur may have been the limiting factor for growth of chicks fed the animal protein concentrates and peas. It did not appear to be the limiting factor in the soybean oil meals or the cottonseed meals. The nutritive values (expressed as grams gain per unit of supplementary protein consumed) used for comparison with the "digestible" organic sulfur were obtained by Evans, Carver, and Draper (5) and Draper and Evans (6) by the gross protein method. In this method chicks are fed a diet containing 8% protein furnished by cereal grains and 3% protein furnished by the protein concentrate being investigated.

The "digestible" organic sulfur content of a protein concentrate is less likely to be the limiting factor in the diet used by Evans, Carver, and Draper (5) and Draper and Evans (6) than it would be in a practical diet or one in which the protein concentrate furnished all of the protein of the diet. The proteins of the mixture of cereal grains used in the gross protein method contained 1.1% "digestible" organic sulfur (Table III) which is equal to that furnished by the best protein concentrates. It is believed, therefore, that the limiting factor under such conditions may be a deficiency of the cereal grain mixture in an amino acid or acids other than the sulfur amino acids. This seems to have been the case when soybean oil meal or cottonseed meal was the protein concentrate.

It has yet to be demonstrated that available cystine and methionine are limiting factors in practical poultry rations when properly heat treated soybean oil meal is the only source of supplementary protein. The results presented here indicate that the diet used by Draper and Evans (6) is not deficient in "digestible" organic sulfur. The protein of this diet contained 1.0% "digestible" organic sulfur when soybean oil meal furnished the supplementary protein. The protein of a chick

starting mash which contained 20% crude protein, 11% from soybean oil meal and 9% from cereal grains, would contain approximately 0.9% "digestible" organic sulfur. The protein of a diet containing only soybean protein, however, would contain only 0.7% "digestible" organic sulfur. Almquist, Meechi, Kratzer, and Grau (12) observed that a diet containing soybean oil meal as the sole source of protein was deficient in available sulfur amino acids. It is possible that a practical ration containing 0.9% "digestible" organic sulfur in the protein would not be deficient in this factor though a soybean oil meal protein containing 0.7% would be deficient. This point is further strengthened by the fact that the protein of the experimental diet (5, 6) contained 1.0% "digestible" organic sulfur and was not deficient in it.

The method outlined in this paper for determining the "indigestible" sulfur appears to be of value in determining the amount of sulfur not nutritionally available. The method was developed to determine whether there were differences in the availability of the sulfur of different soybean oil meals. Though none were found in the meals studied the method appears to be of value in studying animal and vegetable proteins, some of which contain as high as 4.0% sulfur in the indigestible portion of the protein.

Despite the good correlation between the "digestible" organic sulfur and the gain per unit of protein for the animal protein concentrates studied, the author does not at present recommend this method to determine protein quality unless further work is done with other animal products. The chemical protein quality index has been demonstrated to be of value in this connection (5, 11). It must be remembered that the relationship between the "digestible" organic sulfur content of proteins and their nutritive value will only hold where the sulfur amino acids are the limiting factor in the protein.

#### SUMMARY AND CONCLUSIONS

A method is described for determining the sulfur content of the fraction of a protein concentrate not dissolved by digestion with pepsin. The "digestible" organic sulfur of a protein concentrate is defined as the organic sulfur minus the sulfur not dissolved by pepsin. A coefficient of correlation of +0.925 was obtained between the "digestible" organic sulfur of the protein and the gross protein value of

animal protein concentrates. A coefficient of correlation of +0.877 was obtained between the "digestible" organic sulfur and the chemical protein quality index. From the results obtained it appears that the "digestible" organic sulfur may have been the limiting factor for the animal protein concentrates and peas studied, but it was not the limiting factor for the soybean oil meals or cottonseed meals with the type of diet fed.

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# The Determination of Iodine in Rat Thyroids \*

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Received February 7, 1945

## INTRODUCTION

Numerous methods for the estimation of iodine in biological material have been described. In most cases the various methods appear to have been found satisfactory by the respective investigators, but very frequently the results have not been so satisfactory in the hands of others. This is equally true for alkaline fusion methods with intermediate distillation before titration (1, 2) and for acid digestion procedures (3, 4, 5, 6, 7, 8) with distillation before titration.

The method described here was developed for the estimation of iodine in individual rat thyroid glands. The principles involved are essentially those first introduced by Hunter (9). The modifications introduced are distillation of the iodine as HI from the acidified fusion mixture before oxidizing the iodide to iodate followed by colorimetric determination of the iodine by the iodo-starch reaction. Fusion with an excess of potassium hydroxide was found satisfactory for the direct ashing of amounts of organic matter of the order found in an individual rat thyroid. Potassium nitrate was omitted in the fusion, hence there was no formation of iodate with its subsequent difficult recovery as found by Riggs (10). The distillation of the acidified fusion mixture eliminated errors due to the presence of iron and other impurities in the final titration.

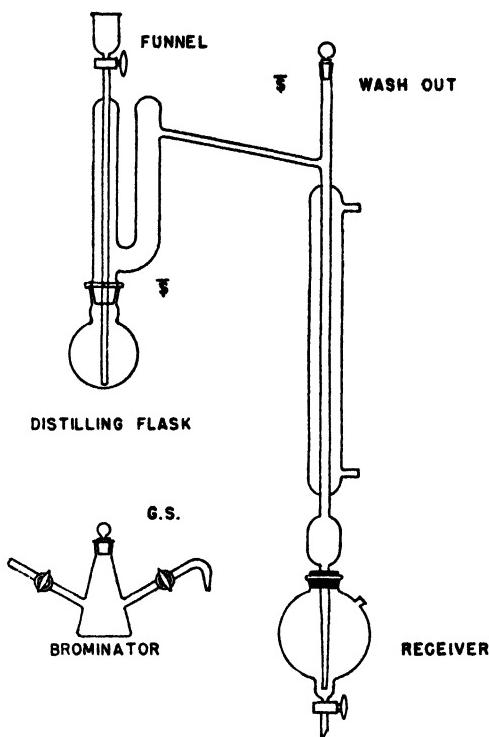
## EXPERIMENTAL

### *Reagents*

Water. Distilled water was redistilled from a potassium carbonate solution in an all-glass still.

\* The data presented in this paper are taken from a Thesis submitted by Virgil L. Koenig to the Faculty of the University of Colorado in partial fulfillment for the degree, Doctor of Philosophy, in 1940.

\*\* Now with Armour Laboratories, Armour and Company, Union Stock Yards, Chicago, Illinois.



Saturated potassium hydroxide. A saturated solution of Baker's Analyzed C.P. potassium hydroxide was made, using the redistilled water.

Fifty per cent sulfuric acid (by weight). 198 ml. of C.P. concentrated sulfuric acid were diluted to 500 ml. with redistilled water.

Three per cent sulfuric acid (by weight). 9 ml. of C.P. concentrated sulfuric acid were diluted to 500 ml. with redistilled water.

Bromine. C.P. bromine was purified by distilling it through a 1 cm. deep layer of 10% copper sulfate in a retort and collecting the bromine in an ice-cooled container.

Starch-iodide indicator. 0.5 g. of soluble starch and 0.5 g. of C.P. potassium iodide were suspended in 10 ml. of redistilled water. This suspension was slowly poured into 40 ml. of boiling redistilled water. This solution was made up every other day.

#### *Procedure*

When it was necessary to keep the rat thyroids for a time before analyses could be made, the thyroids were placed in individual vials containing small amounts of absolute alcohol until analyses could be

made. The rat thyroid and alcohol were placed in a 60-70 ml. nickel crucible, 1 ml. of saturated potassium hydroxide added, and the crucible placed in an oven at 105°C. overnight so that the material evaporated to dryness. The crucible containing the dry material was placed in a cold electric furnace, the temperature of which was gradually raised to 400°C. over a period of about thirty minutes. A slow oxidation of organic matter without spattering is assured by this procedure. The crucible was then removed and allowed to cool. This fused material was transferred to the 50-ml. distilling flask with not more than 25 ml. of redistilled water. A glass bead was added, and the flask connected to the distilling apparatus shown in the drawing. In the receiver was placed enough water to cover the end of the condenser tube. Four drops of 3% sulfuric acid were added to the receiver and then bromine vapor introduced by means of the bromine dispenser of Hays (8). An atmosphere of bromine vapor was always maintained in the receiver. At times it was necessary to add additional bromine vapor in order to replenish the atmosphere above the distillate. Five ml. of 50% sulfuric acid were added to the distilling flask through the separatory funnel sealed in at the top of the distilling apparatus. The material was distilled rapidly over a free flame until fumes of sulfur trioxide appeared in the top of the condenser, care being taken not to flood the side arm. The distilling flask was removed and emptied. The condenser tube was washed with redistilled water through the ground-glass opening at the top. The contents of the receiver were transferred to a 125-ml. Erlenmeyer flask containing a bead and then evaporated on an electric hot plate to about five ml. The flask was then removed and the contents evaporated carefully over a very low free flame to 2 or 3 ml. The material was transferred to a Klett colorimeter tube, the flask washed with small quantities of redistilled water two or three times and the washings added to the colorimeter tube, care being taken to leave sufficient room for the indicator. The colorimeter tube was cooled in running water, which usually was 10° to 15°C. Six drops of the starch-iodide indicator solution were added, the contents made up to the 5 ml. mark, and the color developed compared in the Klett photocalorimeter with a standard curve. This standard curve was determined by adding the indicator to various quantities of a standard biniodate solution and measuring the color developed on the colorimeter. On high iodine values, the contents could be diluted to the 10 ml. mark. When the contents were diluted to 10 ml., an additional six

drops of the starch-iodide indicator solution were added before dilution. In this way the ionic strength of KI in the final test was always maintained at a constant level, and the amount of indicator added provided an adequate excess. Hence, inconsistencies in the production of the starch-iodine color due to variable ionic strengths of KI were obviated.

Determinations were made on known amounts of potassium iodide, to which a small amount of peptone as organic material had been added. Blank determinations on the peptone alone showed it to be free

TABLE I  
(Iodine in form of KI, ashed with 30-40 mg of peptone)

I <sub>2</sub> added in µg.	I <sub>2</sub> recovered in µg	Recovery per cent
2	2.35	117
2	2.15	107
2	2.25	113
2	2.00	100
4	3.95	99
4	3.80	95
6	6.20	103
6	6.05	101
10	9.80	98
10	9.90	99
20	21.00	105
20	19.60	98
20	19.80	99
20	20.80	104

(Following amounts of iodine in form of 7-iodo-8-hydroxyquinoline-5-sulfonate)

8.87	9.65	109
8.87	9.65	109
4.43	4.75	107
4.43	4.70	106

of iodine in the quantities used. The results indicated an average accuracy within 10 per cent for low iodine values of the order of 2 µg. For values above 2 µg. an accuracy within 5% could be assumed. Results of these determinations are given in Table I. Included in the table are results from the analysis of 7-iodo-8-hydroxyquinoline-5-sulfonate at two levels.

Results on the iodine contents of thyroid glands from rats under various conditions of treatment will be reported in a later paper.

Reproducible results were always obtained with the colorimeter. Hays (8) examined a large number of commercial soluble starches and found that any differences in these starches had little or no effect on the reproducibility of results.

### SUMMARY

A method suitable for the determination of iodine in single rat thyroids has been developed which is simple, rapid, and accurate. The method is satisfactory for a range of 1 to 20  $\mu\text{g}$ . of iodine in the presence of 30 to 40 mg. of protein.

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# Spectrophotometric Studies of the Oxidation of Fats \*

## IV. Ultraviolet Absorption Spectra of Lipoxidase-oxidized Fats

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Received February 26, 1945

### INTRODUCTION

The seeds of legumes, especially soybeans, have been shown by several investigators to contain enzyme systems capable of peroxidizing unsaturated fatty acids. The action of this and related enzyme systems on many substrates and in the presence of many inhibitors has been followed by oxygen absorption (1, 2, 3), carotene destruction (4, 5, 6), and peroxide formation (7, 8). In the course of the investigation of the spectra of autoxidized fatty acids (9, 10) it became of interest to learn whether the spectra of lipoxidase-oxidized fatty acids were similar to those of autoxidized fatty acids.

In preliminary experiments (11) it was shown that when a few grams of lard fatty acids in 10 cc. of alcohol were mixed with 80 cc. of 2.5% extract of powdered fat-free soybean meal and aerated for one hour, the absorption spectrum of the fatty acids underwent considerable change. The experiments were repeated on similar quantities of oleates and ethyl linoleate, and changes of spectra accompanied by peroxide accumulation were noted only in the latter case. In an effort to determine which fatty acids are affected by the action of lipoxidase, as judged by changes in absorption spectra, a series of fatty acids and esters have been subjected to lipoxidase action and the results reported.

### EXPERIMENTAL

The ethyl oleate and oleic acid were prepared by Dr. F. L. Greenwood. Linoleic acid, linolenic acid, and arachidonic acid (90% pure), all prepared by low tempera-

\* This work was supported in part by a grant from the National Live Stock and Meat Board.

ture crystallization, were kindly supplied by Dr. J. B. Brown. Elaidolinolenic and conjugated linoleic acids were prepared by Dr. J. P. Kass. Ethyl linoleate was prepared by the method of Rollet (12),  $\alpha$ -eleostearic acid from tung oil (13), pseudo-eleostearic acid and its methyl ester by conjugation of linseed oil fatty acids (14), linolelaidic acid by selenium isomerization of ethyl linoleate (15), and ethyl linolenate from linseed oil (16).

The lipoxidase extract was prepared by suspending 2.0 g. of 300 mesh fat-free soybean meal in 100 cc. of water. The suspension was centrifuged, the supernatant liquid decanted, and stored in the frozen condition. All determinations were made using the same lipoxidase preparation.

The pure substrates were dissolved in 95% ethyl alcohol and one cc. of the substrate solution was pipetted into each of three 10 cc. graduated cylinders equipped with glass stoppers. To the first sample was added distilled water (5.0 cc.); to the second was added 2.0 cc. of lipoxidase preparation and 3.0 cc. of water; and to the third was added 2.0 cc. of boiled extract and 3.0 cc. of water. The three cylinders were shaken periodically and after 10 minutes (30 minutes in the cases of solid substrates) 5.0 cc. of iso-octane was added to each. The samples were shaken vigorously and allowed to settle. The clear iso-octane solutions were then taken for the spectrophotometric determinations with the Beckman spectrophotometer.

The substrate recovered from water was assumed to be unchanged by the treatment, and assuming the extinction coefficients of the pure material in alcoholic solution to be the same as those in iso-octane solution, the concentration of substrate in iso-octane solution could be calculated. The recovery of the substrate by iso-octane from all three samples was assumed to be equal, and thus the results are reported as  $E_{4cm}^{1\%}$  for all three solutions. The light absorption due to substances extracted from the enzyme extract by iso-octane was found to be negligible.

## RESULTS AND CONCLUSIONS

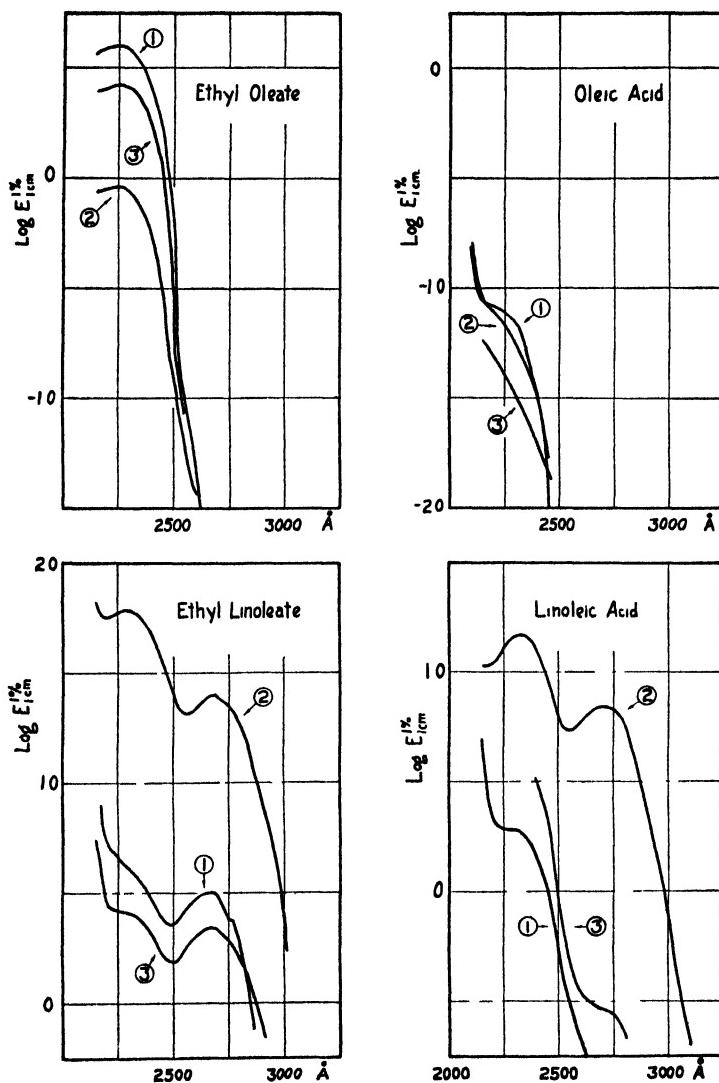
The spectra of the substrate recovered from water (Curves 1), recovered from active lipoxidase preparation (Curves 2), and recovered from boiled lipoxidase preparation (Curves 3) are shown for each of the substrates in the accompanying figures. In the case of pseudo-eleostearic acid, the substrate was dissolved in alcohol containing 5% iso-octane. This change of procedure was made in the hope that the emulsion of iso-octane formed when water is added to the alcohol containing it might carry dissolved substrate. The results were similar when iso-octane was not included in the alcohol. In the figure for methyl pseudoeleostearate, the points A, B, and C indicate the peaks of the curves obtained when iso-octane was included in the solvent alcohol. A, B, and C correspond to Curves 1, 2, and 3, respectively. This comparison indicates that the results are qualitatively the same in the presence or absence of iso-octane.

The decrease in absorption in the region of 2300 Å by ethyl oleate may be ascribed to the action of the lipoxidase upon some impurity present in the ethyl oleate because pure ethyl oleate shows no maximum in absorption at this wave length. The similarity of the spectra of the three samples of oleic acid leads to the conclusion that oleic acid forms no spectrophotometrically detectable products under the action of lipoxidase. Ethyl linoleate and linoleic acid show marked changes in ultraviolet absorption spectrum upon oxidation by lipoxidase. The spectra of the oxidized samples show distinct maxima at 2350 and 2700 Å with increases in absorption of from 5 to 10 times. Linolenic acid and its ethyl ester show the same qualitative changes in spectra after lipoxidase action as do the linoleates. The absorption of methyl arachidonate also increased upon treatment with the active enzyme preparation.

The elaidinized forms of linoleic and linolenic acids, linolelaidic and elaidolinolenic acids, respectively, were found to remain essentially unaltered in the presence of lipoxidase, indicating that although the double bonds exist in the same positions as in the natural forms of these acids, the trans-isomers of the natural acids are not substrates for lipoxidase as judged from absorption spectra. This finding is in agreement with the work of Strain (17) and of Sumner (18) who showed that only the cis configuration is oxidized by lipoxidase.

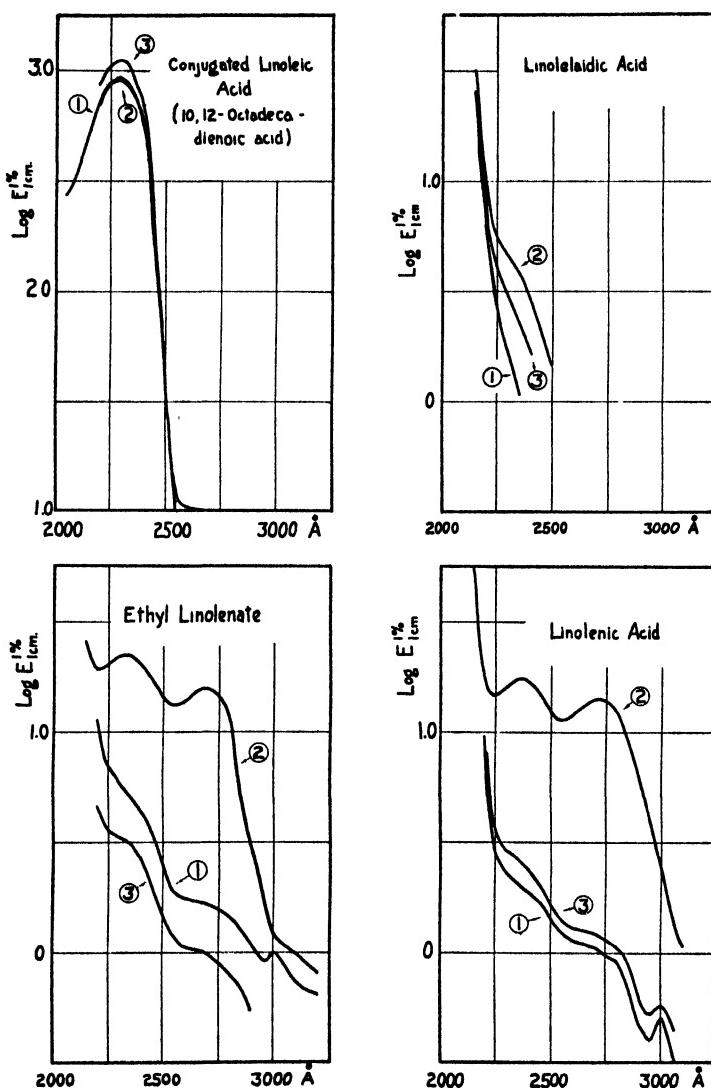
The conjugated form of linoleic acid, 10,12-octadecadienoic acid, was found to remain unaltered after exposure to lipoxidase.  $\alpha$ -Eleostearic acid, naturally occurring 9,11,13-octadecatrienoic acid, showed no marked changes in spectrum after exposure to lipoxidase. This acid has been reported to be partially oxidized by lipoxidase by Söllmann (19) and Sumner (18). Pseudoeleostearic acid, 10,12,11-octadecatrienoic acid, and its ester showed a considerable reduction of absorption in the region of 2700 Å. This change, which takes place with the inactivated enzyme preparation, is accelerated further by the unheated extract. This may indicate that the 9-10 double bond is not required for the action of lipoxidase as was reported by Sumner (18) and Strain (17). It may also indicate that the cis and trans configurations of the  $\alpha$ -eleostearic acid are different from those of the pseudoeleostearic acid.

The work of Söllmann (20) has shown that oxidation of linolenic acid by lipoxidase leads to products capable of forming hydrazones and semi-hydrazones. Peroxides have been detected and measured as end



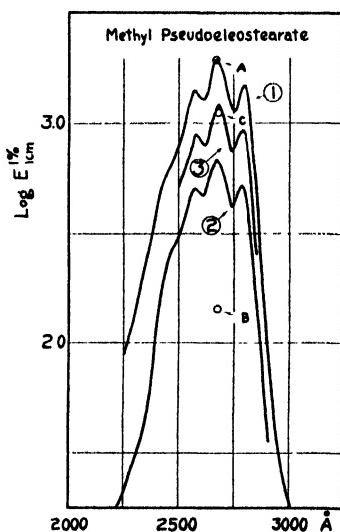
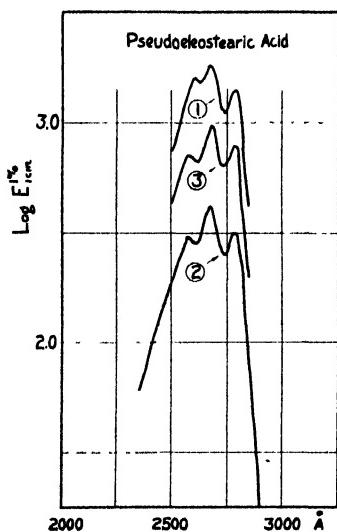
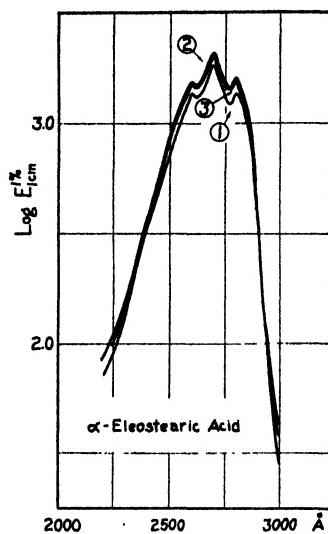
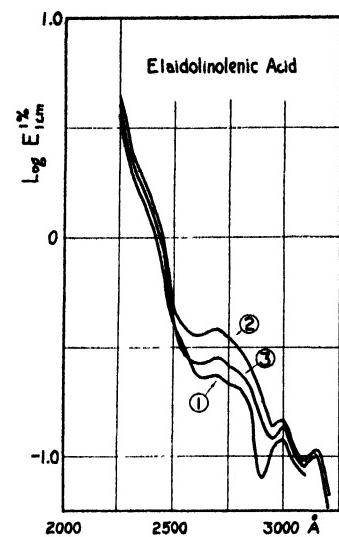
FIGS 1-4

Curves 1—Original Substrate, Curves 2—Substrate plus Active Lipoxidase,  
Curves 3—Substrate plus Inactive Lipoxidase



FIGS. 5-8

Curves 1—Original Substrate; Curves 2—Substrate plus Active Lipoxidase;  
Curves 3—Substrate plus Inactive Lipoxidase



FIGS. 9-12

Curves 1—Original Substrate; Curves 2—Substrate plus Active Lipoxidase;  
Curves 3—Substrate plus Inactive Lipoxidase

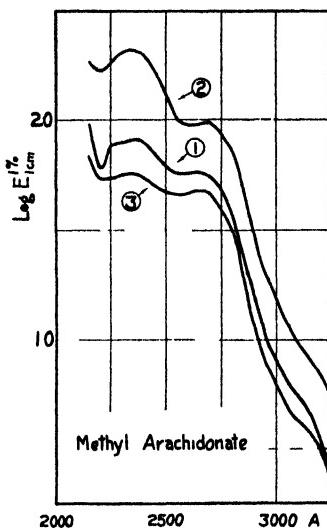


FIG. 13

Curves 1—Original Substrate, Curves 2—Substrate plus Active Lipoxidase;  
Curves 3—Substrate plus Inactive Lipoxidase

products of the oxidation. Peroxides do not show specific absorption bands in the region of the spectrum with which the present study is concerned, and thus could not be responsible for the distinct maxima observed in the oxidized fatty acids (10, 21). Unsaturated carbonyl compounds, however, do show intense absorption near 2300 and 2700 Å and could account for the maxima observed. Conjugated trienes also show triple absorption maxima near 2700 Å, and conjugated dienes show a maximum near 2300 Å. That the band observed in the oxidized fatty acids is not due to the conjugated trienes is indicated by the smooth curve with a single maximum. The similarity of the spectra of lipoxidase-oxidized linoleates, linolenates, and arachidonate indicates that the products formed from these naturally occurring substrates are similar. It is probable that the products which show the two absorption maxima are unsaturated carbonyl compounds in which the carbonyl group and carbon-to-carbon double bonds are conjugated.

The increased spectral absorption produced by the action of active lipoxidase upon the natural fatty acids is so similar to that observed in autoxidation of fats and fatty acids (9, 10, 11) that it seems likely

the same absorption products are produced by both methods of oxidation.

### SUMMARY

1. Using spectrophotometric measurements as a means of detecting changes in substrates, it has been shown that oleic acid, ethyl oleate, conjugated linoleic acid, linolelaidic acid, elaidolinolenic acid, and  $\alpha$ -eleostearic acid are not substrates for lipoxidase.
2. Linoleic acid, ethyl linoleate, linolenic acid, ethyl linolenate, and methyl arachidonate showed increased absorption near 2300 and 2700 Å indicating that these substances were changed by the lipoxidase. Pseudoeleostearic acid and its ester showed decreased absorption near 2700 Å indicating the destruction of the conjugated triene substrate.
3. The products which give the increased absorption in the cases of the naturally occurring substrates are probably conjugated unsaturated carbonyl compounds.

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# **Studies on Growth and Blood Formation in Guinea Pigs \***

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Received February 15, 1945

## **INTRODUCTION**

Work previously reported from this laboratory (1) showed that the addition of 25 per cent of linseed oil meal to a purified ration supplemented with the known vitamins did not render it adequate for growth and survival of guinea pigs, and that the addition of solubilized liver<sup>1</sup> to such a linseed oil meal containing ration (S-44) improved growth and survival. Data accumulated during the past year confirm these observations. Unfortunately, we stated that the addition of solubilized liver to Ration S-44 gave "normal" growth, and considered a growth rate of 4 or 5 g. per day to be "normal" or "excellent." Woolley (2), Woolley and Sprince (3), Hogan and Hamilton (4), and Kuiken, McCoy, Schultze, and King (5) have also uncritically assumed a growth of 3 or 4 g. per day to be "normal" for the guinea pig. It will be evident from the data to be presented here that growth of less than 7 to 8 g. per day from the second to the eighth week of life cannot be considered "normal" if growth on natural foodstuffs is accepted as the standard.

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the Cerophyl Laboratories, Inc., Kansas City, Missouri, and the Research Funds of the University.

We are indebted to Merck and Company, Inc., Rahway, New Jersey, for the synthetic vitamins; to the Abbott Laboratories, North Chicago, Illinois, for haliver oil; to The Wilson Laboratories, Chicago, Illinois, for the various liver products; and to Sharp and Dohme, Inc., Glenolden, Pennsylvania, for sulfaguanidine and succinylsulfathiazole.

<sup>1</sup> Solubilized liver (Fraction L) is that portion of an aqueous liver extract precipitated from solution by addition of ethanol to 70 per cent concentration, then rendered water-soluble by enzyme action, dried and powdered.

In this paper we wish to report further studies on the effect of various supplements and of sulfonamides on growth of and blood formation in guinea pigs kept on a standard basal ration.

#### EXPERIMENTAL

The general procedure has been similar to that previously described (1). Young pigs from our stock colony were placed on experiment when they weighed 150 to 200 g. (8 to 16 days old). They were housed in metal cages on 1/2 inch mesh screens, weighed three times a week, and fed and watered twice a day. The cages, food hoppers, and water bottles were sterilized frequently and were never interchanged. Oral supplements were given with individual, sterilized pipettes and a clean piece of paper was placed on the balance pan before weighing each animal.

Through the kindness of Dr. H. H. Hoyt and Dr. C. E. Blye all animals which died on experiment were autopsied and the organs cultured for bacteria at the Veterinary Disease Control Laboratory of the Wisconsin State Department of Agriculture.

Cell counts and hemoglobin determinations were made in duplicate on blood obtained from the ear of animals fasted 4 to 6 hours. The guinea pigs were immobilized in stout paper or small canvas bags, as suggested by Dodgson, fastened firmly about the animal's neck with a hemostat. Differential leucocyte counts, when done, were made in the counting chamber, and only the proportion of polymorphonuclear leucocytes present was determined. The non-polymorphonuclears were almost entirely lymphocytes, although occasional monocytes were seen.

The basal ration used consisted of the following: sucrose 72 g., casein 20 g., salts IV (6) 4 g., corn oil 4 g., thiamine 0.3 mg., riboflavin 0.5 mg., pyridoxin 0.4 mg., Ca *d*-pantothenate 1.5 mg., nicotinic acid 5 mg., choline chloride 100 mg., biotin 0.02 mg., *t*-inositol 50 mg., and 2-methyl-1,4-naphthoquinone 0.2 mg. This ration (S-29c) differs from Ration S-29 used in previous work (1) in that somewhat increased levels of certain of the vitamins were used and biotin, inositol and vitamin K were included. Borden's Labco casein was substituted for the ethanol-extracted reprecipitated casein used in S-29 except in certain groups receiving Rations S-44 and S-45 as mentioned later. S-44 and S-45 are used to designate rations containing 25 per cent

linseed oil meal, and 25 per cent linseed oil meal + 4 per cent solubilized liver, respectively, and do not specify the type of casein or the vitamin supplement. The letter *c* following the ration number indicates that ration contains Labco casein and the vitamin supplement described above. When the basal ration was supplemented with appreciable amounts of dry material, the sucrose content was correspondingly decreased. Rations were mixed weekly and stored in the refrigerator. The stock ration was a commercial pelleted guinea pig feed containing ingredients such as alfalfa leaf meal, wheat, oats, and soybean oil meal. The proximate analysis is given as protein 17.5 per cent, carbohydrate 58 per cent, fat 2.5 per cent and fiber 9 per cent.

All animals were given 20 mg. of ascorbic acid orally every other day and 1 mg. of  $\alpha$ -tocopherol and 0.05 cc. of haliver oil orally twice a week. The solution of  $\alpha$ -tocopherol in corn oil was mixed with the haliver oil just prior to feeding.

The "folic acid" concentrate was prepared from solubilized liver by the method of Hutchings, Bohonos, and Peterson (7). It was adsorbed on, and eluted from, successively, norite, Superfiltrol, and norite, and was fed at a level equivalent in "folic acid" content to 4 per cent of solubilized liver as determined by assay with *Streptococcus lactis* R.<sup>2</sup> Growth promoting activity for *Lactobacillus casei*<sup>2</sup> was concentrated to a similar degree.

### *Inadequacy of Rations Containing Linseed Oil Meal and Solubilized Liver*

Table I summarizes results obtained since our earlier paper (1).

#### *1. Growth*

The growth on the stock ration was slightly greater than that reported by Glimstedt (8), which is the best growth that has been recorded. With a summer diet of hay, grain, carrots, rutabagas and grass, Glimstedt found that guinea pigs between the second and eighth weeks of life gained 7.2 g. per day.

That 25 per cent of linseed oil meal did not render the purified basal ration adequate was appreciated earlier (1), but it is easily seen that although 4 per cent of solubilized liver improves this diet, it falls far short of producing the growth obtained with the stock ration. The

<sup>2</sup> The folic acid assays were kindly performed by Lester T reply.

TABLE I \*

*Growth and Blood Status of Guinea Pigs Fed Natural Rations and Purified Rations Containing Linseed Oil Meal Alone and With Various Supplements*

Ration	Growth		Blood			
	4 Weeks	6 Weeks	3 Weeks		6 Weeks	
			g. per day	per mm <sup>3</sup>	Leuco- cytes	Hemo- globin
Stock ration	15	15	7.4 5.3 9.9 $\sigma = 1.2$	7.5 5.0-10.2 $\sigma = 1.2$	4800 2800-6200	13.6 13.0-14.5 $\sigma = 1900$
S-44c (S-29c + 25% linseed oil meal)†	61	32	2.2 -1.6-5.8 $\sigma = 0.9$	3.0 0.8-6.3 $\sigma = 1.2$	2000 600-5300 $\sigma = 1300$	11.3 9.5-14.9 $\sigma = 1.2$
S-44c + "folic acid" concentrate	25	8	1.8 0.6-4.5	1.5 1200-5200 $\sigma = 1200$	2500 8.3-12.3 $\sigma = 1.2$	10.4 8.3-12.3 $\sigma = 1.2$
S-44c + 4% solubilized liver (S-45c)††	26	23	4.1 0.9-7.7 $\sigma = 1.4$	4.0 1.4-7.8 $\sigma = 1.6$	1900 700-3000 $\sigma = 800$	11.4 9.6-13.1 $\sigma = 0.9$
S-44c + 6% liver 1:20 powder	10	10	5.7 3.2-7.7 $\sigma = 1.4$	5.7 3.9-6.9 $\sigma = 1.1$	6000 5500-8000	12.8 12.1-13.3 $\sigma = 2.500$
S-44c + 16% whole liver substance	3	3	4.7 4.1-5.5	5.1 4.8-5.3		4800 2700-6000
S-45c + 16% alfalfa leaf meal	4	3	6.3 5.4 7.7	6.5 5.8-6.9		3000 2500 4000
S-45c + 8% brewers' yeast	4	3	5.6 4.3-7.2	6.4 4.8-7.9		2000 1900-2100
S-45c + 8% grass juice powder	3	3	6.6 5.4-8.2	7.0 5.7-8.3		
S-45c + 8% corn steep powder	3	3	5.0 3.9-6.0	5.5 4.2-6.5		

\* The figures in each group are arranged thus:

number of animals

AVERAGE

range

standard deviation ( $\sigma$ )

† Includes animals receiving Ration S-44 (1).

†† Includes animals receiving Ration S-45 (1).

animals receiving S-44 alone made an average daily gain of 3.0 g. as compared with -0.6 g. for the 23 animals previously reported. One animal of the present series grew 6.3 g. per day and 5 others grew in excess of 4 g. per day as compared with a maximum of 2.0 g. per day at six weeks for one guinea pig of the earlier group. Survival on Ration S-44 remained poor, only 19 out of the 51 animals lived 6 weeks on experiment. The improvement in the growth of the survivors could not be correlated with the type of casein used nor with changes in the vitamins supplied, but occurred shortly after receiving a new shipment of linseed oil meal. The linseed oil meal used in the earlier work had been stored for nearly two years. Various samples of linseed oil meal have been tried, but none has produced the consistent failure seen with the original supply.<sup>3</sup> Another variable is the animals. The stock colony has been developed with animals which have survived on experimental rations. This procedure may select animals which are able to grow and survive on purified diets either because of genetic factors affecting metabolism, or, more probably, because of an intestinal flora capable of synthesizing the missing dietary essentials. The behavior of animals which survive 6 weeks or longer suggests that intestinal synthesis plays an important role. After growing at a slow rate for 2 or 3 weeks, these animals show an improved growth and may continue to grow 4 or 5 g. per day for several weeks, most of them eventually plateauing and dying after a brief period of weight loss.

The growth on Ration S-44 + 4 per cent solubilized liver (S-45) was slightly less than that reported previously (4.3 g. per day), and 6 of 26 animals failed to survive 6 weeks. The decrease in growth may be due to the substitution of Labco for the cruder ethanol-extracted reprecipitated casein. The 6 animals which received the latter grew 4.3 g. per day while the 14 supplied with Labco casein averaged 3.7 g. per day. However, this difference is less than the standard deviation of either group.

When a "folic acid" concentrate was substituted for the solubilized liver all beneficial effect was lost and the animals appeared, if anything, worse off than on Ration S-44 alone. Of 11 animals allowed to remain unsupplemented, except for the "folic acid" concentrate, for 6 weeks, only one survived.

<sup>3</sup> The linseed oil meal now used is a special lot of solvent extracted meal obtained from Archer-Daniels-Midland Co., Minneapolis, Minnesota.

Other forms of liver have been tried as supplements to Ration S-44c. More uniform and better growth has been obtained with whole dried liver substance and liver 1:20 powder (dried crude water extract of liver).

In an attempt to achieve the growth obtained with the stock ration a number of materials were added to Ration S-45c. Of these 8 per cent of grass juice powder,<sup>4</sup> 8 per cent of corn steep powder,<sup>5</sup> 16 per cent of alfalfa leaf meal, and 4 per cent of brewers' yeast gave definite increases in growth rate.

## 2. *Hematology*

White cell counts and hemoglobin values for guinea pigs on various diets are given in Table I. Animals receiving the stock ration are included here as a standard, as they were in the studies on growth.

The blood of guinea pigs fed the stock ration contained 6,100,000 red cells (average of 27 counts on 6 animals) and an average of 5000 white cells per mm<sup>3</sup>. The mean hemoglobin content was close to 14 g. per 100 cc. These figures closely approximate the averages compiled by Scarborough (9) in his review of the composition of the blood of guinea pigs (RBC 5,750,000, WBC 10,770, Hemoglobin 90 to 100 per cent on the Sahli scale), except for the numbers of leucocytes. However, all the white cell counts included in Table I are from animals 8 weeks old or less. Four adult guinea pigs taken from the stock colony where they had received stock ration + greens had an average white count of 11,000 cells per mm<sup>3</sup>. Several authors, cited by Scarborough, have shown that young guinea pigs have only 1/2 to 1/5 as many leucocytes per unit volume of blood as adults. There is apparently little change with age in the erythrocyte count or in the hemoglobin concentration. The hemoglobin values found in animals on stock ration are surprisingly constant. The lowest value encountered was 13 g. per 100 cc.

In contrast to the animals receiving the stock ration, guinea pigs on Rations S-44 and S-45 showed a marked leucopenia and a definite anemia, although there was considerable individual variation. No corre-

<sup>4</sup> Grass juice powder is the spray-dried pressed juice of cereal grasses. One g. of grass juice powder is equivalent to about 4 g. of dried grass (Cerophyl).

<sup>5</sup> Corn steep powder is spray-dried corn steep water, supplied through the kindness of the American Maize Products Company, Roby, Indiana.

lation appeared between the degree of leucopenia and the severity of the anemia in an individual, but animals which grew poorly generally had the higher hemoglobin values. With Ration S-44 the type of casein did not affect the growth or leucocyte count but hemoglobin formation was poorer in those animals which received the Labco casein (10.5 g. per 100 cc. at 3 weeks and 10.8 g. per 100 cc. at 6 weeks). Weekly blood studies showed that animals kept on Ration S-44 developed anemia and usually leucopenia at some time during the experiment, although these conditions were not always clearly evident at the 3 or 6 week period. All the guinea pigs which had hemoglobins of 12 g. per 100 cc. or over at 3 or 6 weeks eventually showed levels less than 12 g. per 100 cc. if they survived. However, these, together with the other animals which survived more than 6 weeks on Ration S-44, often showed a remission of the leucopenia and anemia, sometimes associated with a spurt in growth. The presence of 4 per cent of solubilized liver (S-45) appears to decrease the anemia slightly but had little or no influence on the leucopenia. The type of casein seems to be without effect when solubilized liver is added.

The cruder preparations of liver offer a great contrast to solubilized liver. Six per cent of liver 1:20 powder raises the white count to the level found in animals on stock ration and lessens the anemia. Sixteen per cent of whole liver substance in 3 animals restored not only the white count but brought the hemoglobin within the range of "normal" values. The measurements on these animals showed less variation and there were no animals with severe anemia (less than 11.0 g. per 100 cc.). The significance of these higher levels of formed elements in the blood is enhanced by the superior growth on these same supplements. It is evident that to maintain even the same level of formed elements in the blood, hemopoiesis must be greater in the more rapidly growing animal.

The addition of "folic acid" concentrate to Ration S-44c did not reproduce the effects of any of the liver preparations. The animals showed the same degree of anemia and leucopenia exhibited by guinea pigs on unsupplemented S-44 with Labco casein.

Of the substances which were tried as supplements to Ration S-45c for their growth effect, data on the blood are available only for alfalfa and brewers' yeast. Sixteen per cent of alfalfa nearly abolished the anemia in the three animals on which it was tried but appeared to have little effect on the leucopenia. Four per cent of yeast increased

neither red nor white cells above the levels usually occurring with solubilized liver in the ration.

In the leucopenia observed all types of leucocytes appear to be affected. There seems to be no significant relation between the differential count and the type of ration fed. Twenty-seven counts over a period of 6 weeks on 6 animals given the stock ration show a low proportion of polymorphonuclear leucocytes, 32 per cent. Marked variation occurs, *e.g.*, in one animal these ranged from 10 to 32 per cent. Thirty-seven animals, at the time they were placed on experiment, had an average of 20 per cent (6 to 38 per cent) polymorphonuclears. After 3 weeks, 7 animals on Ration S-44 averaged 21 per cent (0 to 55 per cent) and 10 animals on Ration S-45 averaged 25 per cent (7 to 45 per cent) polymorphonuclear leucocytes.

#### *Effect of Succinylsulfathiazole*

In an attempt to control the effects of intestinal synthesis and achieve the degree of difference between linseed-oil-meal-containing rations with and without liver that had obtained in earlier work, succinylsulfathiazole was added to a number of rations (Table II).

Succinylsulfathiazole, 1/2 per cent, mixed with the ground stock ration did not affect growth or blood formation adversely. Added to Ration S-44c, succinylsulfathiazole caused cessation of growth after 2 weeks. The only animal which survived 3 weeks showed very severe anemia and leucopenia.

The presence of 4 per cent of solubilized liver (S-45c-ss) did not overcome the effect of succinylsulfathiazole. The animals grew for a longer period but eventually ceased to grow, lost weight for 3 to 5 days, and died.

Other forms of liver increased survival and prolonged the period of growth but ameliorated only slightly the anemia and leucopenia. It should be noted that on Ration S-45c-ss + liver 1:20 powder the hemoglobin concentration of both survivors reached 13 g. per 100 cc. at 7 weeks. The white count was slightly lower (2400) than at 6 weeks.

Alfalfa leaf meal and dried grass (Cerophyl) improve growth and prolong survival but are less effective than liver fractions for blood formation. Soybean oil meal and corn steep powder were the least effective supplements tried, although even these appeared to increase growth and survival over Ration S-45c-ss. Since the addition of one part of ground stock ration to two parts of Ration S-45c-ss gives

yeast nucleic acid, biotin, xanthopterin, and adenine-thiomethylpentoside.

B. *p*-Aminobenzoic Acid Plus Biotin Therapy. *p*-Aminobenzoic acid plus biotin had no growth-stimulating effects in rats fed 1% sulfaguanidine (Group 4, Series A) and yielded variable growth responses in animals fed 0.5% sulfasuxidine (Group 4, Series C). In the latter group, four of the seven rats showed an average gain of 16 g. per rat

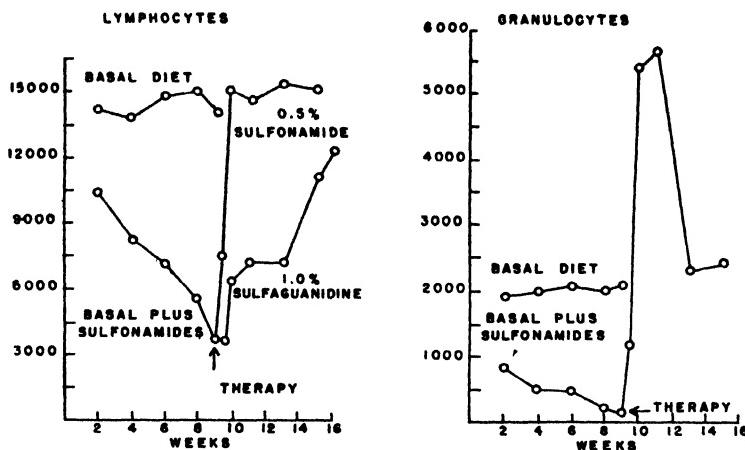


FIG. 2

#### Leukopenia on Sulfonamide-Containing Diets and Leukopoietic Effects of Whole Liver, and Norit Eluate Plus Biotin

Ordinates designate the number of cells per cmm. of blood. Curves previous to therapy represent the combined average of rats receiving 1.0% sulfaguanidine and 0.5% sulfaguanidine or sulfasuxidine (Series A, B, and C). Therapy refers to whole liver, or norit eluate plus biotin. Granulocyte response to therapy is the combined average for all rats receiving the sulfonamides (Groups 5 and 6, Series A; Group 1, Series B; Group 5, Series C).

during the ten day period of therapy. No growth effects were noted in the remaining three rats of this group.

The administration of *p*-aminobenzoic acid plus biotin to rats receiving either 1% sulfaguanidine or 0.5% sulfasuxidine resulted in similar variable and delayed leukocyte responses. In five of the fourteen rats, granulocyte counts between 1200 and 7000 per cmm. were noted after one to two weeks of this treatment. A similar granulopoietic

number of leukocytes ranged from 2000 to 4000 and the number of granulocytes varied from 0 to 400 per cmm. A moderate anemia (Av. hb. = 10 g./100 cc.) was observed after nine weeks in rats receiving 1.0% sulfaguanidine. Hemoglobin determinations were not made in Series B and C.

After nine weeks, treatment was instituted as indicated in Table I. The animals continued to receive their respective sulfonamide-contain-

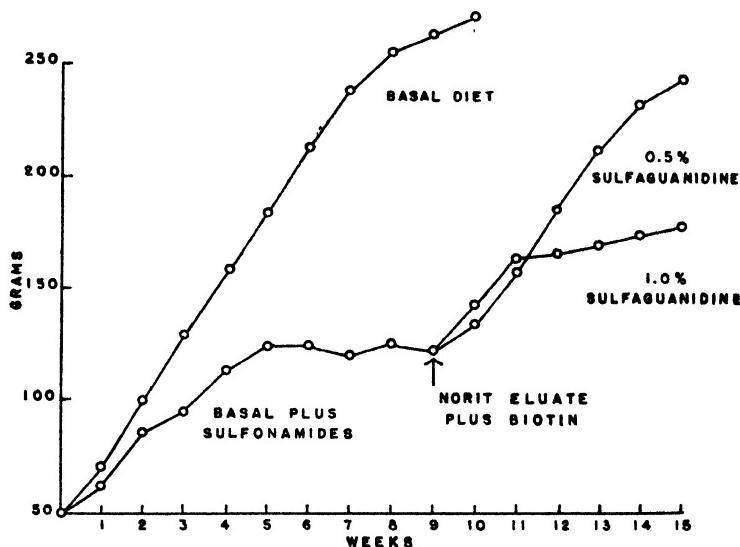


FIG. 1

Growth Inhibition on Sulfonamide-Containing Diets and the Growth-Stimulating Effect of Norit Eluate Plus Biotin

Curve previous to therapy represents the combined average of rats fed 1.0% sulfaguanidine and 0.5% sulfaguanidine or sulfasuxidine (Series A, B, and C).

ing diets during the administration of the various substances. Daily leukocyte counts were generally made in the two days immediately preceding therapy and at intervals thereafter as indicated in Fig. 2.

*A. Substances with No Therapeutic Activity.* The following substances were completely ineffective in counteracting the growth inhibition or leukopenia in animals fed the sulfonamides: nicotinic acid, adenine, synthetic batyl alcohol, glutamine, pimelic acid, uracil, guanine, xanthine, yeast adenylic acid, muscle adenosine triphosphate, *i*-inositol,

*L. casei* assay procedure of Landy and Dicken (5) using as a reference standard a liver concentrate, furnished by Parke, Davis and Co., which had been previously standardized against crystalline vitamin B<sub>c</sub>.

Xanthopterin was synthesized <sup>2</sup> according to the method of Purmann (6). In some experiments xanthopterin received from W. A. Lott of the Squibb Institute for Medical Research was used. Identical results were obtained with both samples.

Methyl acetamide (acetyl methylamine), an Eastman Kodak product, was fractionally distilled and the fraction boiling between 206.4 and 207.4°C. at 745 mm. of Hg was employed.

*p*-Chloro-xylenol (1,3-dimethyl-2-chloro-5-hydroxybenzene) was prepared by the chlorination of 1,3-dimethyl-5-hydroxybenzene with sulfonyl chloride and purified by recrystallization from ligroin (m.p. 113.2°-114.2°C., uncor.).

Adenosine triphosphate was prepared from rabbit muscle by a modification of Lohmann's procedure (7) as used by Cori.<sup>3</sup>

Blood studies were performed on blood from tail vessels. Hemoglobin was determined by the method of Evelyn (8) and total leukocyte and differential counts were carried out in accordance with standard procedures. Two hundred to 400 cells were usually counted in the differential determinations, but as many as 700 cells were counted when the percentage of granulocytes was very low.

## EXPERIMENTAL

### I. Effects of Various Substances on the Growth Inhibition and Leukopenia Caused by Sulfonamides

In this experiment, weanling rats were fed the basal diet modified by the inclusion of 1% sulfaguanidine (Series A), 0.5% sulfaguanidine (Series B) and 0.5% sulfasuxidine (Series C) for nine weeks prior to therapy. Thirty-five weanling rats, serving as controls, received the basal diet throughout the course of the experiment. The animals were weighed weekly and blood studies were performed biweekly.

The growth and leukocyte picture of the controls were normal as shown in Figs. 1 and 2. During the nine-week period the effects noted in the various groups receiving the sulfonamides (Series A, B, and C) were similar and will be discussed together. The growth of these animals was retarded during the first five weeks on experiment; the growth curve plateaued at an average weight of 125 g. for four weeks thereafter (Fig. 1). The administration of the sulfonamides resulted in a gradual decrease in the number of circulating white blood cells; a marked leukopenia was noted after nine weeks (Fig. 2). At this time the total

<sup>2</sup> We are indebted to Dr. K. F. Swingle for this preparation.

<sup>3</sup> Dr. G. T. Cori (Personal communication).

# The Toxic Effects of Sulfaguanidine and Sulfasuxidine and Their Therapy in Rats Receiving Purified Diets

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Received November 9, 1944

## INTRODUCTION

In a preliminary report (1), we have described the effects of various substances upon the leukopenia and granulopenia resulting from the oral administration of sulfaguanidine to rats receiving a purified diet. Marked leukopoietic activity was exhibited by whole liver and by a norit eluate fraction of liver. In the present paper, we wish to describe in greater detail the effects of these and other substances, particularly methyl acetamide, upon the leukopenia and growth inhibition noted in rats receiving either sulfaguanidine or sulfasuxidine in a purified diet.

Pathological changes in animals utilized in the present study have been described elsewhere (2).

## METHODS AND MATERIALS

Male, weanling, albino rats from the Sprague-Dawley colony were employed in these studies and were housed in individual cages with wide-mesh screen bottoms.

The basal ration had the following composition: sucrose, 76; Labco casein, 18; salts (3), 4; corn oil, 2; and 2-methyl-1,4-naphthoquinone, 0.001. Modifications of the basal ration were made by substituting the desired substance for an equal weight of sucrose. All diets were fed *ad libitum*. The following amounts of the B vitamins were fed daily in supplement dishes to all rats: thiamine, 30 γ; pyridoxin, 30 γ; riboflavin, 30 γ; calcium pantothenate, 100 γ; and choline chloride, 10 mg. Two drops of haliver oil containing 1.3 mg. of *dl*-α-tocopherol acetate were given weekly to each rat.

The norit eluate fraction, containing 20 mg. of solids per cc., was prepared from Wilson's Liver Extract L according to the directions of Hutchings, *et al.* (4). One cc. of this fraction contained the equivalent of 3.6 γ of vitamin B<sub>c</sub> as determined by the

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TABLE II  
*Urobilinogen plus Urobin Excretion in Urine and Feces after  
 Sulfaguanidine Ingestion*

Sub- ject	Urine (mg./day)						Feces (mg./day)					
	1st Day	2nd Day	3rd Day	Average First 2 Days	Aver- age 3 Days	1st Day	2nd Day	3rd Day	Average First 2 Days	Aver- age 3 Days		
T	1.07	1.2	2.5	1.13	1.59	134	168	219	151	173		
Y	0.96	1.30	2.2	1.13	1.49	157	155	211	156	176		
M	1.92	2.15	2.0	2.02	2.03	233	247	220	240	233		
D	1.09	1.16	2.8	1.12	1.68	197	141	162	169	166		
K	0.72	0.98	2.1	0.85	1.26	199	196	208	197	201		
L	2.6	.23	3.0	2.45	2.60	224	230	250	227	234		
C	1.46	1.52	2.71	1.49	1.89	180	161	237	170	192		
B	1.12	1.92	2.87	1.52	1.97	151	216	241	183	202		

### DISCUSSION

Inability to demonstrate any decrease in urobilin excretion in cases M and L is not easily explained. The possibility that organisms responsible for the conversion of bilirubin to urobilinogen were refractory to the anti-biotic action of the sulfaguanidine must be considered. The comparatively short depression of urobilin excretion (production?) which lasted not more than 48 hours may be attributed, in part, to the metabolic adjustments which the organisms made. The fact that no decreased urobilin appeared in the excrement of cases M and L speaks for the normal excretory function of the liver as far as urobilin metabolism is concerned. It is interesting that little change could be observed in the color of the feces in all the subjects. Steigmann and Dyniewicz (6) claim that "after administration of sulfonamides an increase in urobilinogen content of both urine and stool was noted in a small number of patients." No information as to dosage or type of sulfonamide given is mentioned. However, it is their belief that the increased excretion of urobilinogen was due largely to an increased blood destruction as a result of therapy.

### CONCLUSION

Six out of eight male adults showed a distinct decrease in urobilin plus urobilinogen excretion in the feces and urine after receiving sulfaguanidine.

The possible mechanisms for these findings are discussed.

The findings in Table I indicate that all the subjects showed normal urine and fecal urobilinogen and urobilin excretory values over a three day period.

At the end of the seventy-two hour control period all the subjects were given 2 g. of sulfaguanidine every three hours for fifteen consecutive hours. The total dosage amounted to 10 g. The usual activity and diet was maintained. At the completion of the twenty-four hour drug intake interval (no specimens were obtained at this time), the collection of the total urine and fecal specimens were resumed. This extended over another seventy-two hour period with urobilinogen and urobilin determinations made on fecal and urine specimens as in the control period. Because the first, and in most cases the second day as well, showed a decreased excretion of urobilinogen plus urobilin in the feces and urine in six of the eight subjects, the average figures of the forty-eight as well as the seventy-two hour value are presented in Table II.

It appears quite obvious from Table II that there is a decided decrease in the fecal and urine urobilinogen plus urobilin values in six of the eight subjects. This is particularly apparent when the first twenty-four hour and forty-eight hour period is compared to that of the control period. Since no statistically important change was evident, the icteric indices values before and after the ingestion of the drug were omitted. The re-establishment of normal urobilinogen and urobilin excretion appears to begin about forty-eight hours after the start of drug administration. The most rapid decline in excretion is obviously in the first twenty-four hours in those who showed any change.

TABLE I  
*Urobilinogen plus Urobilin Excretion in Urine and Feces of Eight Normal Subjects over Three Day Period*

Sub- ject	Urine (mg./day)						Feces (mg./day)					
	1st Day	2nd Day	3rd Day	Average First 2 Days	Aver- age 3 Days	1st Day	2nd Day	3rd Day	Average First 2 Days	Aver- age 3 Days		
T	1.2	1.9	2.2	1.55	1.76	180	210	240	195	210		
Y	3.0	2.5	2.6	2.75	2.70	184	219	191	201	198		
M	2.0	2.5	1.7	2.25	2.06	240	226	200	233	222		
D	3.1	2.4	2.2	2.56	2.75	191	216	211	203	206		
K	2.5	1.8	3.0	2.17	2.44	188	191	190	189	187		
L	2.0	1.7	2.5	1.85	2.60	217	200	234	208	217		
C	2.6	2.4	2.3	2.50	2.43	206	224	220	215	216		
B	2.1	3.0	2.1	2.55	2.73	198	234	224	216	218		

# The Effects of Sulfaguanidine on Urobilinogen and Urobilin Metabolism

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Received July 12, 1944

## INTRODUCTION

Systemic effects in laboratory animals have been observed when their intestinal flora has been affected by the administration of the relatively insoluble antibiotic sulfaguanidine. Block, McKibbin, and Elvehjem (1), Daft, Ashburn, and Sebrell (2), as well as Welch, Mattis, and Latven (3), and later, Welch and Wright (4), have presented sufficient proof that a marked change may occur in the production of certain essential metabolites when experimental animals are fed sulfaguanidine. For example, the decrease in prothrombin time in rats was noted when animals were fed sulfaguanidine (4). This of course suggested interference with synthesis of Vitamin K by the normally existing groups of organisms in the intestinal tract of the rat.

It is generally accepted that bilirubin is normally reduced to urobilogen in the intestine by bacterial action. Part of the urobilin is reabsorbed into the portal circulation and part is excreted in the feces and urine. The metabolism of urobilinogen in humans receiving sulfaguanidine seemed interesting enough to warrant investigation.

## EXPERIMENTAL

Eight normal male subjects between the ages of 26 to 42 years were chosen. For three consecutive days all their urine and fecal specimens were collected according to the technique advocated by Watson (5). Watson's quantitative method for feces and urine urobilinogen and urobilin was used (5). Table I (control period) presents the daily as well as the average excretion for the forty-eight and seventy-two hour periods in the eight subjects. Both fecal and urine estimations are presented. The diet was controlled to the extent that only overcooked meats were permitted. Fresh fruits, vegetables, milk products, and carbohydrates made up the 2800 calorie daily intake complete.



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TABLE III  
*Effect of Extraction Method upon the Apparent Nicotinic Acid Content of Dehydrated Foods*

Dehydrated Food	Nicotinic Acid Content	
	Acid Hydrolyzed γ per g.	Enzyme Digested* γ per g.
White Potato	48.2	23.4
	35.3	9.0
	51.8	16.2
Sweet Potato	14.0	12.1
	16.0	14.0
Turnip	57.0	25.0
Onion	6.9	6.8
Carrot	18.5	11.3
	17.0	10.0
Beet	8.1	7.2
Cranberry	7.3	7.1
Tomato	53.0	54.0

\* Enzyme digestions were carried out with takadiastase and papain (13).

### SUMMARY

Improvements in the microbiological assay for nicotinic acid are described, using *Lactobacillus arabinosus* 17-5 as the test organism. The medium has been simplified by the inclusion of nicotinic acid-free extracts (Lloyd's reagent) of natural materials. These increase the response of the organism to the added vitamin.

Good agreement of assay values at different levels and good recoveries of added nicotinic acid are obtained for a wide variety of materials.

A comparison of the nicotinic acid found in dehydrated foods after enzyme and acid digestion is given.

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TABLE II  
*Nicotinic Acid Assays and Recoveries*

Material <sup>a</sup>	Sample mg.	Nicotinic Acid Found			Nicotinic Acid per gram			Recovery, per cent		
		I* γ	II* γ	III* γ	I γ	II γ	III γ	I	II	III
Dried pork muscle	0.25	0.059	0.060	0.058	236	240	236			
	0.5	0.127	0.119	0.119	254	238	238			
	0.75	0.194	0.179	0.180	258	239	240			
	1.0	0.253	0.236	0.228	253	236	228			
	0.25+.05 γ nicotinic acid	0.112	0.110	0.108				106	100	100
	0.5+.10 γ nicotinic acid	0.223	0.216	0.215				96	97	96
Skim milk powder	5	0.039	0.040	0.040	7.8	8.0	8.0			
	10	0.086	0.081	0.084	8.6	8.1	8.4			
	15	0.131	0.120	0.128	8.7	8.0	8.4			
	20	0.170	0.158	0.174	8.5	7.9	8.7			
	5+.05 γ nicotinic acid	0.079	0.088	0.093				80	96	108
	10+.10 γ nicotinic acid	0.172	0.169	0.185				86	88	101
Whole wheat flour	0.83	0.045	0.041	0.042	55	49	51			
	1.66	0.094	0.080	0.087	58	48	52			
	2.5	0.135	0.113	0.126	54	45	50			
	3.3	0.165	0.148	0.161	50	45	48			
	0.83+.05 γ nicotinic acid	0.108	0.093	0.092				126	106	100
	1.66+.10 γ nicotinic acid	0.192	0.172	0.177				98	92	90

<sup>a</sup> These samples were obtained from Dr. F. M. Strong of the University of Wisconsin, as part of a series receiving collaborative study.

\* I Snell and Wright medium.

II Krehl, Strong, and Elvehjem medium.

III Present authors' medium.

the troublesome "drifts" which are sometimes encountered with the Snell-Wright medium have been largely eliminated.

The values obtained for some materials are the same whether the sample is digested with acid, alkali, or enzymes. This is particularly true of fresh foods (13). However, most dehydrated foods must be hydrolyzed with acid (or alkali) in order to liberate maximum amounts of nicotinic acid (Table III). Enzyme digestion with takadiastase and papain releases less than half of the total vitamin present in some of the dehydrated foods. Whether the total nicotinic acid which is measured after these hydrolyses represents the amount available for human nutrition is unknown at present.

*Norite Treated Yeast Extract.* 10 g. of yeast extract (Difco) are steamed 10 minutes in 200 ml. of water. After cooling, the pH is adjusted to 1.5 with concentrated HCl solution and 10 g. of Norite are added. The mixture is shaken 20 minutes, filtered, the pH is readjusted to 1.5, and the adsorption is repeated. The filtrate is neutralized to pH 6.6, steamed, cooled, and filtered. One ml. of the final solution is equivalent to 50 mg. of the original yeast extract.

The above adsorption procedure is essentially that of Neal and Strong (11). It yields a nicotinic acid-free extract which is also free of pantothenic acid, and which has been used as an ingredient of the basal medium for the assay of the latter vitamin (11, 12).

The remaining components of the medium are prepared according to the method of Snell and Wright (1).

### *Preparation of Extracts*

Samples to be assayed are hydrolyzed with 1 N  $H_2SO_4$  according to the method of Krehl, *et al.* (2). It has been customary in this laboratory, however, to filter the extracts after neutralization, prior to using them for assay.

### *Assay Procedure*

Assays are performed in 20  $\times$  150 mm. lipless Pyrex test tubes. Test samples at pH 6.6-6.8 are diluted to contain approximately 0.06  $\gamma$  of nicotinic acid per ml. Four tubes are used for each sample, with the volume of test solution usually varying from 1 to 4 ml. A standard set of 8 tubes is included in each rack. These contain 0.0, 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, and 0.50  $\gamma$  of nicotinic acid, respectively. The dilute standard, containing 0.10  $\gamma$  of nicotinic acid per ml., is prepared daily from a stock solution kept in 35% ethanol in a refrigerator. The contents of each tube are diluted to 5 ml. with water, and 5 ml. of the basal medium (Table I) are added. The tubes are plugged, autoclaved 10-15 minutes at 15 pounds pressure, cooled and inoculated with 1 drop of the dilute inoculum already described. The tubes are then incubated in a water bath at 37° for 40 to 72 hours, and the acid produced is titrated with alkali.

## RESULTS

Table II contains the assay values and recoveries of added nicotinic acid for three materials of widely varying potency. Comparable assay values are obtained among the three methods used, although the two modified media provide the best agreement of values at the four levels assayed and the best recoveries of the added vitamin. In further experiments with the present medium, the addition of nicotinic acid to eleven other materials has given recoveries of  $102 \pm 10$  per cent. Agreement of values at different assay levels has been similarly good throughout the testing of approximately two hundred samples, and

5 mg. of *untreated* yeast and liver extracts are added. On the present medium, the organism produces 1.38 ml. of 0.1 *N* acid per 0.05  $\gamma$  within the preferred assay range (0.05 to 0.30  $\gamma$ ). This is a higher value than is obtained with the other two media (0.84 ml. and 1.13 ml. per 0.05  $\gamma$ , respectively), although the data presented by Krehl, *et al.* indicate a response of 1.40 ml. per 0.05  $\gamma$ . Our attempts to duplicate the high values obtained by these workers have been unsuccessful. The cause of the discrepancies is not known, although the standard curve obtained by Barton-Wright (3), with a medium similar to that of Krehl, *et al.*, shows a response of 1.08 ml. per 0.05  $\gamma$ , which is in reasonable agreement with our value for this medium.

The desirable low blank values are best obtained when heat sterilization is kept to a minimum. In several experiments prolonged heating has produced increases of approximately 0.5 ml. in the blank readings. Acid production at other points of the curve is not increased correspondingly, but coincides with the usual curve beyond the 0.2  $\gamma$  level. The reason for this rise is not clearly understood. Bovarnick (10) has obtained small yields of nicotinamide by prolonged heating of asparagine or glutamine with various amino acids. However, the autoclaving of asparagine with foodstuffs in the present medium failed to produce measurable stimulation of growth.

#### *Preparation of Stock Solutions*

*Hydrolyzed Casein.* 100 g. of "vitamin-free" casein are autoclaved 16 hours at 15 pounds pressure with 500 ml. of 25% sulfuric acid. The pH is adjusted to 3.0 with Ba(OH)<sub>2</sub>. The solution is diluted to approximately 2 liters, filtered, shaken 20 minutes with 10 grams of Norite, and filtered again. The pH is readjusted to 3.0, and the charcoal adsorption and filtration are repeated. Most of the excess sulfate is removed with Ba(OH)<sub>2</sub>, and the solution evaporated in *vacuo* to 1000 ml. A final pH adjustment is made to 6.6. The solution, containing 100 mg. of casein per ml., is steamed, cooled, filtered, and stored under toluene.

*Lloyd's Reagent Treated Peptone, Liver and Yeast Mixture.* 25 g. of Bactopeptone, 10 g. of liver extract (Lederle or Wilson) and 10 g. of yeast extract (Difco) are autoclaved 15 minutes with 400 ml. of 1 *N* NaOH solution. The solution is cooled, acidified to pH 0.8-1.0 with concentrated HCl solution, diluted to 500 ml., and 45 g. of Lloyd's reagent are added. The mixture is agitated 20 minutes and centrifuged. The supernatant liquid is decanted, the pH is readjusted to 0.8-1.0, and the adsorption is repeated twice. Eighteen grams of K<sub>2</sub>HPO<sub>4</sub> are added and the pH is adjusted to 6.6. The extract is steamed, cooled, and filtered. Each ml. is thus equivalent to 50 mg. of peptone, 20 mg. of liver extract, and 20 mg. of yeast extract. The solution is stored under toluene in a refrigerator.

was decided not to include extra adenine in the basal medium. The effect is not noticed below a level of 50  $\gamma$  of extra adenine per tube, and it is unlikely that test solutions would supply this amount. Yeast, which is rich in purines, gives excellent assay values and recoveries of added nicotinic acid on the regular basal medium.

### *Response to Nicotinic Acid*

The growth curve using the present medium is compared in Fig. 1 to those obtained in this laboratory with the media of Snell and Wright

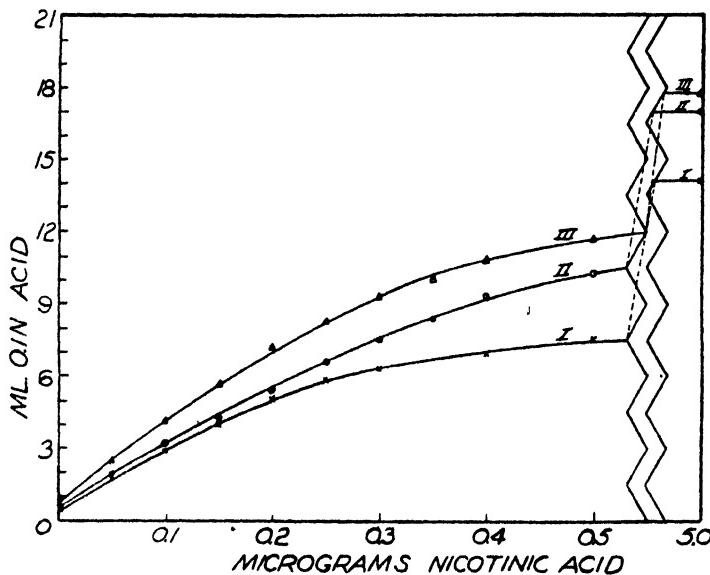


FIG. 1  
Acid Production by *L. arabinosus* with Various Media (72 Hours)

- I. Snell and Wright
- II. Krehl, Strong, and Elvehjem
- III. Present authors

and of Krehl, *et al.* All titration values have been converted to ml. of 0.1 *N* acid. Low blank values are observed with all three media. The two modified media permit high acid production, although the highest figure (17.8 ml. with 5  $\gamma$  of nicotinic acid in the present medium) is somewhat short of the 20.7 ml. produced in either medium when

TABLE I  
*Composition of the Basal Medium*

Glucose	40 g.
Sodium acetate, anhydrous	36 g.
Hydrolyzed casein, vitamin-free	10 g.
Lloyd's reagent treated	{ Peptone Liver extract Yeast extract
	5 g. 2 g. 2 g.
Norite treated yeast extract	2 g.
Cystine	400 mg.
Calcium pantothenate	200 γ
Inorganic salts A, B(1)	10 ml. each
Distilled water to 1000 ml., pH 6.6-6.8	

not only by the casein hydrolyzate but also by the peptone, liver and yeast extracts. The casein digest may therefore be treated thoroughly with charcoal to remove the nicotinic acid. Commercial "vitamin-free" caseins may be hydrolyzed without preliminary purification. The hydrolyzate will not support optimum growth in the Krehl-Strong-Elvehjem medium, but it is quite satisfactory when used with the other extracts in the present medium.

The use of peptone, liver and yeast extracts permits omission of tryptophan, purines, and all the vitamins except pantothenic acid from the medium. Lloyd's reagent is preferred to charcoal for removal of nicotinic acid from these extracts as it is more selective in its action of the low pH employed. The extracts thus prepared increase the response of the organism to nicotinic acid. They are supplemented with a charcoal treated yeast extract which slightly further improves the growth of the organism.

The complete basal medium could not be improved either by changing the concentration of the ingredients or by adding all of the other supplements found in the synthetic type media. A mixture of pyridoxal and pyridoxamine<sup>2</sup> was likewise without effect.

Adenine produces an unusual effect upon the growth curve for nicotinic acid, either when added alone or together with guanine and uracil. In the presence of relatively large amounts of this purine (100 to 500 γ per tube in addition to the quantity furnished by the natural extracts in the medium) the lower portion of the curve is made steeper, whereas the upper slope is depressed. In view of this depression, it

<sup>2</sup> Samples of pyridoxal and pyridoxamine were kindly furnished by Dr. E. E. Snell of the University of Texas.

The second approach, which has been followed in the present work, is to prepare a medium containing natural extracts which are treated to remove the vitamin being assayed. Unknown stimulatory substances are thus retained which are not present in the synthetic media above. These increase the response of the organism to the added vitamin and tend to overcome the extra growth stimulation by the materials being tested.

Lloyd's reagent has been used successfully for the quantitative adsorption of nicotinic acid (8, 9). In the present method this reagent has been used to remove the vitamin from peptone, yeast and liver extracts. These extracts have been incorporated into a basal medium which has been found reliable for the routine assay of nicotinic acid. The number of ingredients in the medium is small, and the response to the added vitamin is greater than may be obtained using previously developed methods.

## EXPERIMENTAL

### *Cultures and Inoculum*

*Lactobacillus arabinosus* 17-5 is carried on yeast agar stab cultures as described by Snell and Wright (1). Inocula are prepared by direct transfer from a suitable stock culture to a tube containing 5 ml. of the basal medium diluted to 10 ml. with 1 γ of nicotinic acid, 5 mg. of liver extract (Lederle or Wilson)<sup>1</sup> and 5 mg. of yeast extract (Difeo). After incubation at 37° for 16 to 24 hours, 1 ml. of the cell suspension is diluted with 15 ml. of 0.9% saline. One drop of the resulting dilute suspension is added to each test culture for assays.

An inoculum of maximum viability is obtained when it is grown rapidly with yeast and liver extracts for approximately 18 hours. When necessary, the grown culture may be stored in a refrigerator for periods up to 24 hours prior to inoculation of assay tubes. Inoculum cultures have been used after 40 hours incubation, but these give poorer growth curves and their regular use is not recommended.

### *Basal Medium*

The optimum concentrations of glucose and acetate for growth of *L. arabinosus* have been determined by Krehl, Strong, and Elvehjem. Their recommendations have been followed successfully in the present work.

The basal medium selected has the composition shown in Table I. Amino acids and related substances (excepting cystine) are supplied

<sup>1</sup> We are indebted to Dr. T. H. Jukes of Lederle Laboratories and Mr. S. W. Hier of Wilson Laboratories for samples of these extracts.

# **Microbiological Assay Methods for Nicotinic Acid \***

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Received March 15, 1945

## **INTRODUCTION**

Microbiological assays of nicotinic acid, using *Lactobacillus arabinosus* as the test organism, have been employed in many laboratories using the original method of Snell and Wright (1) or the improved method developed by Krehl, Strong and Elvehjem (2). The present communication describes further changes which appear to constitute improvements over both of these methods. A third modification by Barton-Wright (3) has appeared recently which is similar to the method of Krehl, *et al.*

Several difficulties were encountered when the Snell-Wright medium was employed. First, as pointed out by previous workers (2, 3), the standard curve was linear over only a very limited range. Second, on this medium the organism was incapable of producing maximum amounts of acid, even in the presence of as much as 5 γ of nicotinic acid per 10 ml. of culture. Third, large "drifts" were often observed among values calculated at progressively higher assay levels, and recoveries of the added vitamin were frequently unsatisfactory.

Improvements in growth media for microbiological assays have been made in two ways. The first is to incorporate all known growth factors into media in which the only unknown ingredient is hydrolyzed vitamin-free casein (2-7). These media permit fairly good growth but are relatively difficult to compound, and care must be taken to prevent contamination or deterioration of the components.

\* A preliminary report was presented before the Chemistry Division at the third meeting of the Oregon Academy of Science, Portland, January, 1945.

Published with the approval of the Monographs Publication Committee, Oregon State College. Research paper No. 89, School of Science, Department of Chemistry.

This work was supported by the Nutrition Foundation, Inc.

The authors wish to acknowledge the technical assistance of Margaret Jean Bennett.

mixture, however, showed an activity approaching that of the protein preparations when considered on a weight basis. However, their activities at optimum concentrations were always considerably below that of the protein preparation.

The general character of the concentration-activity curves of the two classes of substances differ considerably and leads one to suspect that a simple prosthetic group-protein relation may not explain the activities of the active phospholipid and lipoprotein assuming an enzymatic mechanism. More definite experiments with this in mind are in progress and will be published subsequently.

#### SUMMARY

1. A quantitative method of assay of thromboplastin substances is described using recalcified dog plasma.
2. Protein and lipid preparations of varying degrees of purity were assayed. The protein preparations were more active than the lipid preparations, and the crude products more active than the purified substances.
3. The lipid preparations reach an optimum activity which falls off at high concentrations in contrast with the protein preparations which maintain a plateau at high concentrations.
4. The active phospholipid fraction is soluble to some extent in 95% ethyl alcohol.
5. The antioxidant, hydroquinone, has been found to protect the phospholipid from oxidation and thus prevent loss of activity.

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TABLE III  
*The Stability of Phospholipin Emulsion*

Days	0.3%		0.1%	
	Room Temp.	37°C.	Room Temp.	37°C.
0	67	67	66	66
12	68	66	66	61
21	68	69	65	65
39	69	64	64	56
56	68	66	59	39
69	71	68	66	39
76	69	61	59	47
88	64	70	57	49
111	70	67	55	22

progressive decrease in activity, the decrease being much more marked with those ampules kept at 37°C. The great variability in the assay results for the 0.1% emulsion incubated at 37°C. is probably due to different rates of deterioration of the material in the individual ampules.

The decrease in activity of the 0.1% emulsion in contrast to the stability of the 0.3% emulsion is understandable when one considers the character of the concentration-activity curve for this material. The 0.3% emulsion is above the optimum concentration and, if a small proportion of the material had deteriorated, it would not be reflected in change of activity.

An interesting observation as a result of this experiment was the marked stability of the material to heat. Sterilizing at 100°C. for 45 minutes did not inactivate the material. Other experiments, however, indicate that longer periods of heating definitely decreases the activity of the preparations.

#### DISCUSSION

The thromboplastin protein agents were extremely potent at their optimum concentrations. The activities of the preparations, however, cannot be compared with the highly purified lipoprotein of Chargaff, *et al.* (1942), since different methods were used in their assay.

A relatively potent lipid preparation can be isolated if precautions are taken to prevent loss of active material during the ethyl alcohol extraction and to protect it from auto-oxidation. Our most active lipid preparations were crude mixtures of cephalin and lecithin. This

TABLE II  
*Activity of 4 Thromboplastic Agents Determined by 2 Methods*

Preparation	Maximum Decrease in Coagulation Time per cent	50% Decrease in Coagulation Time mg./cc.
Thromboplastin Suspension	85	0.065
Lipoprotein	89	0.092
Phospholipin	65	0.123
Cephalin	56	0.937

plastin suspension was the most active, a concentration of 0.065 mg. per cc. being necessary to produce a 50% decrease in coagulation time. Since only 0.2 cc. of the solution is added for this assay, this would represent a total of only 0.013 mg. of dry material. The lipoprotein preparation, although slightly more active at optimum concentrations, was not as active on a weight basis. The lesser activity of the more highly purified lipoprotein may be due either to loss of some potent constituent or to some denaturation of the protein. For the phospholipin, a concentration of 0.123 mg. per cc. was required to produce a 50% decrease in coagulation time, while the cephalin preparation was the least active, requiring 7 times as much material to produce the same effect. This agrees with the work of Chargaff, *et al.* (1942) that the more active phospholipin fractions are to a certain extent soluble in 95% ethyl alcohol.

#### STABILITY OF PHOSPHOLIPIN EMULSION

The stability of phospholipin emulsion was investigated to determine whether an emulsion of this material would retain its thromboplastic activity over a long period of time.

An emulsion of phospholipin in 0.9% NaCl was prepared in two concentrations, 0.1% and 0.3%. The material was filtered, sealed in 2 cc. ampules, and sterilized for 45 minutes at 100°C. Some tannish material settled out but emulsified readily on shaking. Ampules of each concentration were divided into two groups. One group was kept at room temperature (20–25°C.) and the other group incubated at 37°C. At various intervals over a period of 111 days an ampule was selected at random from each group and assayed. The results of these assays are presented in Table III.

The results indicate that the 0.3% emulsion maintained its activity over the period tested. In the case of the 0.1% emulsion there was a

concentrations, after which there is a leveling off as the concentration increases. The phospholipin also produces a sharp rise in activity at low concentration but reaches a plateau and further increase in concentration decreases the activity. In the case of cephalin, there is a gradual rise in activity at low concentrations which reaches a peak, and any further increase in concentration decreases the activity. This decrease is more apparent in the case of cephalin than in the case of phospholipin. Hanzlik, *et al.* (1920) reported similar results with cephalin preparations. This phenomenon might be due to either (1) a

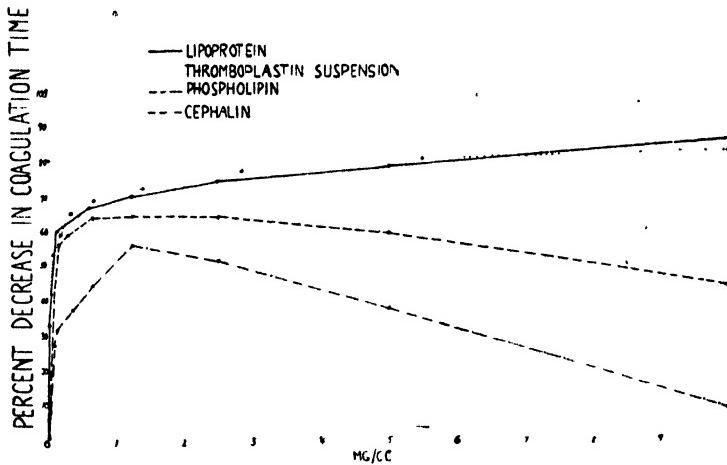


FIG. 1  
Concentration-Activity Curve of Various Thromboplastic Agents

general colloidal effect of high concentrations of lipid emulsions, or (2) to a lowering of pH due to the interaction of calcium ion and the high concentrations of cephalin as shown by Wadsworth, Maltaner, and Maltaner (1931). This is evidenced by the fact that if a solution containing 20 mg. of phospholipin per cc. of saline is added, there is a definite precipitation of some of the plasma constituents.

The activities of the preparations may be measured by two methods: either by comparing the maximum per cent decrease in coagulation time, or by determining the concentration of material necessary to produce a 50% decrease in coagulation time.

Table II demonstrates that the protein preparations were more active than the lipid preparations. On a weight basis, the thrombo-

3. *Phospholipin.* Phospholipin is the term used to designate a lipid preparation containing both cephalin and lecithin. The components are not separated as it is thought that the more active cephalin fractions are soluble in ethyl alcohol and will be lost with the removal of lecithin. Phospholipin is isolated by a modification of the procedure outlined by Folch (1942). The tissue is macerated and extracted three times with four volumes of acetone. The residue is extracted twice with two volumes of petroleum ether and the petroleum ether fraction evaporated to dryness in *vacuo* at 45°C. The residue is taken up in ethyl ether, 200 cc. per kg. of tissue, and placed in the refrigerator overnight. The precipitate of sphingomyelin and cerebroside is removed by centrifugation and the supernatant ethyl ether solution evaporated to 150 cc. per kg. of tissue. The material is placed in a refrigerator for three hours and then centrifuged to remove the precipitate. The supernatant ethyl ether solution is evaporated to 100 cc. per kg. of original tissue and again cooled for 8 hours in the refrigerator. The slight precipitate which forms is removed by centrifugation. Five volumes of acetone are added slowly with stirring to the supernatant ethyl ether solution to precipitate the phospholipids. The precipitate is removed by filtering through a Büchner funnel, washed with acetone and desiccated under high vacuum.

Phospholipin, when first prepared, is granular and has a slight yellowish tinge, but within a week the dry material darkens and finally becomes reddish-brown. Concomitant with the darkening a decrease in activity occurs. Since the darkening of the preparation might be due to oxidation of the unsaturated fatty acids, we attempted to prevent this oxidation by the use of antioxidants. Studies were carried out with thiourea, ascorbic acid, acetone bisulfite, and hydroquinone. Of these agents, hydroquinone was by far the most effective, although the others protected the material to some degree. A 15% concentration of hydroquinone was effective in protecting a phospholipin preparation for several months. Studies of this phase of the work are still in progress and will be reported later.

4. *Cephalin.* The cephalin is prepared by making a 10% solution of phospholipin in ethyl ether and then adding this solution to five volumes of 95% ethyl alcohol which precipitates the cephalin and leaves the lecithin in solution. The precipitate is washed with alcohol and dried on a Buchner funnel.

#### ACTIVITIES OF PREPARATIONS

As the thromboplastin suspension was already liquid, the amount of material per cc. was determined by drying at 110°C. The lipoprotein was not soluble in saline and a suspension was made by mechanical agitation. The phospholipin and cephalin readily formed stable emulsions when shaken.

Successive geometric dilutions of the material were made with saline solution to obtain concentration-activity curves. The activities expressed as per cent decrease in coagulation time are presented in Fig. 1 as a function of the concentration.

It will be seen from Fig. 1 that the thromboplastin suspension and lipoprotein preparations produce a sharp rise in activity at low

TABLE I  
*Values for Different Assays on the Same Sample of Cephalin*

Date of Assay	<i>Tc</i>	<i>Tt</i>	Decrease in Coagulation Time per cent
11-19-43	120	60	50
11-24-43	195	90	54
11-26-43	375	189	50
12- 6-43	345	165	52

are presented in Table I. It will be noted that there is considerable variation in the values for the control coagulation time. We believe that this is due to differences in obtaining the blood. It has been our experience that when any difficulty is encountered in withdrawal of the blood the coagulation time is decreased due to release of thromboplastin by the injured cells.

#### PREPARATION

Four different preparations from beef brain were assayed, two containing protein and two free from protein as determined by the xanthoproteic test.

1. *Thromboplastin Suspension.* The thromboplastin suspension represents a crude saline extract which contains much extraneous matter. Beef brains are stripped of superficial blood vessels and membranes and macerated in four times their volume of 0.9% NaCl. A few drops of 10% alcoholic thymol are added as a preservative and the mixture placed in the refrigerator overnight. The material is then pressed through several layers of gauze. The filtrate is a suspension containing approximately 44 mg. of solid material per cc. and is preserved with additional alcoholic thymol.

2. *Lipoprotein.* The lipoprotein represents a more highly purified preparation of the active agent in thromboplastin suspension and is prepared by the isoelectric precipitation method of Cohen and Chargaff (1941). Brain tissue is macerated and extracted with twice its volume of 0.9% NaCl overnight in a refrigerator. The mixture is then filtered through gauze and centrifuged to remove suspended material. The supernatant liquid is adjusted to pH 5.2 with 10% acetic acid and the protein precipitate collected by centrifugation. The precipitate is dissolved in water by bringing the pH to 8.8 with *M* KOH and is again centrifuged to remove insoluble material. The isoelectric precipitation is repeated and the precipitate redissolved in water at pH 8.8. The slight residue is removed by centrifugation. The solution is dialyzed in running tap water overnight and finally in distilled water. The solution is frozen with a mixture of solid CO<sub>2</sub> and acetone and then evaporated to dryness under high vacuum. The yield was 45 mg. of lipoprotein per 100 g. of original brain tissue. Using the method of Cohen and Chargaff (1940) to split off the lipids, the material was found to contain 15.7% lipids.

of the thromboplastic activities of protein and lipid preparations using a standardized procedure of assay.

#### METHOD OF ASSAY

To determine the thromboplastic activities of the various preparations, an assay method had to be developed which would yield consistent and quantitative results. The following procedure was found to fulfill the necessary requirements.

From an unanesthetized dog, arterial or venous blood is withdrawn into a syringe containing 0.13 M sodium citrate, the ratio being 1 part citrate to 9 parts of blood. The citrated blood is immediately centrifuged for 45 minutes at 2500 r.p.m. and the upper three-fourths of the plasma carefully removed. In small test tubes, 10 by 75 mm., 0.6 cc. of the citrated plasma is placed, followed by 0.2 cc. of 0.9% NaCl. At this point, 0.25 cc. of 1.0% CaCl<sub>2</sub>·6H<sub>2</sub>O is added and the tube immediately inverted to insure thorough mixture. At various intervals thereafter the tube is inverted and the time required for the formation of a solid clot noted by means of a stop-watch. The amount of CaCl<sub>2</sub> solution added to subsequent tubes varies by increments of 0.02 cc. until that point is reached at which the coagulation time becomes independent of the amount of CaCl<sub>2</sub> added. In practice this amount is usually between 0.25 and 0.30 cc. of 1.0% CaCl<sub>2</sub>·6H<sub>2</sub>O. That amount of CaCl<sub>2</sub> which gives a minimum coagulation time is then used in the assay of thromboplastin preparations.

The coagulation time of tubes containing only plasma, NaCl, and CaCl<sub>2</sub> is called the control coagulation time (*Tc*). The same amount of CaCl<sub>2</sub> is then added to tubes containing 0.6 cc. of plasma and 0.2 cc. of the thromboplastin in saline and the coagulation time determined. This is called the thromboplastin coagulation time (*Tt*). By subtracting this coagulation time from the control coagulation time, the decrease in coagulation time due to the thromboplastin is obtained. By dividing this value by the control coagulation time and multiplying by 100, we obtain the per cent decrease in coagulation time. Thus,

$$\% \text{ decrease in coagulation time} = \frac{Tc - Tt}{Tc} \times 100.$$

In the assay, the calcium ion is always added in excess so that the formation of thrombin becomes independent of the calcium concentration at those high levels. Thus, inadvertent addition of excess calcium due to the thromboplastin preparations will not be reflected in the activity. Since the activity of the thromboplastin is expressed in terms of a control which has the same amount of prothrombin, the effect of the prothrombin level of the blood is nullified.

It was found that a constant temperature bath was not necessary, as the values of per cent decrease in coagulation times were independent of the temperature over a range of 6°C. to 30°C.

To demonstrate the consistency of results obtained by this method of assay, the values for different tests on the same sample of cephalin

# The Activities of Various Preparations of Thromboplastic Substances

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Received March 1, 1945

## INTRODUCTION

Preparations of substances having thromboplastic activity fall into two categories, those containing protein and those free from protein. The preparations containing protein are usually saline extracts of various tissues known to be rich in thromboplastic activity (Maltaner and Maltaner, 1943) and invariably contain a great deal of extraneous material. Chargaff, *et al.* (1942), however, isolated from saline extracts of beef brain by ultracentrifugation an apparently homogeneous macromolecular lipoprotein which was very potent as a thromboplastic agent.

Many investigators have shown that a lipid having thromboplastic activity could be isolated from tissue (Bordet and Delange, 1913; McLean, 1916). From solubility studies it was found that the lipid had the properties of the phospholipid cephalin. While the lipid preparations free from protein had considerable activity (Chargaff, *et al.*, 1936), they were not as active as the more potent protein preparations (Mills, 1921).

By determining the degree of unsaturation in a cephalin preparation and concurrently measuring its activity, McLean (1917) came to the conclusion that the activity of the phospholipid was proportional to the degree of unsaturation of its fatty acids. Chargaff, *et al.* (1942) similarly concluded that the thromboplastic activity resides in the more highly unsaturated, alcohol-soluble cephalin fractions.

Since different procedures were used to assay the activity of various types of preparations, no quantitative comparison could be deduced from the data. It was the purpose of this study to make a comparison

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## SUMMARY

Guinea pigs on a ration of crude natural foodstuffs grow 7.5 g. per day from the second to eighth weeks of life and their blood averages 5000 leucocytes per mm<sup>3</sup> and 14 g. of hemoglobin per 100 cc. Growth and survival on purified rations, supplemented with linseed oil meal and solubilized liver, are poorer than on the crude diet and the animals develop anemia and leucopenia.

Succinylsulfathiazole does not affect growth or blood formation on the crude ration, but abolishes growth and induces severe anemia and leucopenia in animals receiving purified rations supplemented with linseed oil meal and solubilized liver. The effect of various supplements on the growth and blood status of animals given linseed-oil-meal-containing purified rations, with and without succinylsulfathiazole, is described.

The relationship of the leucopenia to infection is discussed.

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measure the poor survival of animals on purified rations. Animals with low white counts were subject to infection with *Salmonella typhi-murium*,<sup>6</sup> or a closely related species, as shown by cultivation of this organism from the liver or spleen after death. Waisman, Rasmussen, Elvehjem, and Clark (10) have reported a similar situation in regard to bacillary dysentery in deficient monkeys. The studies of Meyer (11) indicate that guinea pigs harbor *Salmonella* more or less constantly in the intestinal tract and that an active infection, characterized by ulceration of the colon, stomach or intestines, invasion of the blood stream, and lodgement of organisms in liver and spleen, usually occurs only when animals are subjected to unfavorable conditions. It seems fairly certain that the stomach ulcers reported by Kohler, *et al.* (12) were caused by this or a similar infection. Following an outbreak of salmonellosis in the stock colony, rigorous sanitation seems to have largely eliminated *Salmonella* infection even in animals on deficient rations. In the absence of *Salmonella*, common intestinal bacteria reach the liver, possibly because of weakened defenses, so that the number of positive cultures remains about the same. *Escherichia coli* is the organism most commonly found except in the succinylsulfathiazole-fed guinea pigs in which *Pseudomonas aeruginosa* (*Bacillus pyocyanus*) predominates.

The absence of any deleterious effect from the addition of succinylsulfathiazole to the stock ration indicates that its effect with purified rations is due to its action on the intestinal bacteria. With purified rations the effect of this drug is different in the rat and guinea pig. Four per cent of solubilized liver does not counteract 1/2 per cent of succinylsulfathiazole in the guinea pig whereas 1 per cent of solubilized liver gives complete restoration of growth and leucocyte count in the rat (Ransone and Elvehjem, 13). Anemia, which is severe in the guinea pig, is not usually seen in the succinylsulfathiazole-fed rat. Although most of the supplements tried with Ration S-45c-ss have improved growth, they have had less effect on blood formation at the levels fed. We will probably not be able to tell until bacteria-free guinea pigs are available whether the amelioration of the deficiency by certain supplements is due to supplying directly substances required in the metabolism of the guinea pig, or is caused indirectly through influence on the enteric bacteria.

<sup>6</sup> Kindly identified by Dr. C. E. Blye.

replaced by cellulose and additional casein. Liver is still required to supply GPF-3. In his original paper (2) Woolley found that GPF-2 could be replaced by 10 per cent of solubilized liver, and in the later work there is nothing to show that the same growth would not have occurred in animals receiving solubilized liver, or the lead acetate norit fraction therefrom plus folic acid, had the cellulose and extra casein been omitted. Growth in all their animals, 3 to 4 g. per day, is such as to indicate that the ration is still inadequate; however, this work does question the indispensability of linseed oil meal.

Employing the rations described by Woolley and Sprince (3), we have repeated and confirmed this work except that the animals supplemented with only the "folic acid" concentrate survived and grew (Table III), whereas the 5 animals reported by Woolley and Sprince did not.

Included in Table III for comparison are growth figures of animals fed various sucrose rations, containing 20 or 30 per cent of casein, with and without liver or cellulose. It is interesting to note that 30 per cent of casein in the basal (S-28) does not help the growth or survival as compared with 20 per cent of casein (S-29c). The addition of 15 per cent of cellulose prolongs survival, but this effect is eliminated by the inclusion of 1/2 per cent of sulfaguanidine. The addition of 4 per cent of solubilized liver to Ration S-29c gave a growth stimulus of short duration. The growth at 6 weeks is about that reported for a larger series of animals on Ration S-29 plus 4 per cent of solubilized liver in an earlier paper (1). Increasing the liver to 8 per cent produces growth only slightly less than that obtained on Ration G-1 + 8 per cent of liver, evidence that with this amount of liver, cellulose and additional protein are unimportant. With 8 per cent of solubilized liver alone, growth approaches that produced by liver plus linseed oil meal (Ration S-15, Table I). However, Kuiken, *et al.* (5) failed to obtain appreciable growth by supplementing a purified basal ration with 10 per cent of liver 1:20 powder. Although we are uncertain whether liver will or will not replace linseed oil meal entirely it is clear that neither one nor both, in the amounts used at present, satisfies the requirements of guinea pigs on purified rations. Perhaps it should be noted that 25 per cent of linseed oil meal provides about 2.5 per cent of fiber, while Woolley's diets contain eight times this amount.

The leucopenia discovered by our blood studies explains in some

TABLE III  
*Effect of Protein, Cellulose, and Liver on Growth and Survival of Guinea Pigs Receiving Purified Rations*

Ration	Growth		Survival days
	4 Weeks <i>g. per day</i>	6 Weeks <i>g. per day</i>	
S-28*	20 -1.7 -2.7-0.1	1 -1.7	20 24 9-46
S-28 +15% cellulose†	8 -0.4 -3.0-1.3	4 -1.0 -2.4-0	8 44 28-58
S-28 +15% cellulose +1/2% sulfaguanidine	12 0	0	12 21 7-37
G-1‡ (glucose, 20% cellulose)	4 6.3	1 5.5	4 25 19-44
G-1 + "folic acid" concentrate	4 2.6 1.6-3.4	3 2.6-4.5	4
G-1 +8% solubilized liver	4 3.8 2.7-4.9	4 3.6 2.8-5.0	4
S-29e	4 0.5 -0.2-1.2	2 0.8	4 36 8-82
S-29e +4% solubilized liver	4 3.4 2.0-4.4	3 1.9 -0.8-3.7	4
S-29e +8% solubilized liver	3 2.8 2.1-3.5	1 3.1 3.0-3.4	3

\* Ration S-28 differs from Ration S-29e in containing 30 per cent of casein and different levels of vitamins. As described in an earlier paper (14) the amount of choline is incorrect. It was 40, not 400 mg. in both rations S-27 and S-28.

† Cellu Flour obtained from Chicago Dietetic Supply House.

‡ Glucose 45 g., Labco casein 30 g., cellulose 20 g., salts 5 g., and vitamins (Woolley and Sprince, 3).

necessary to evaluate such an interpretation. We recognize that the additive effect of different supplements may be due to increasing the amount of one or a few essentials rather than supplying many separate factors.

Woolley and Sprince (3) have recently considered that the factor in the 50 per cent ethanol extract of linseed oil meal (GPF-1) is "folic acid" while the activity of the extraction residue (GPF-2) can be

the factors needed for optimal nutrition with Ration S-44. Four per cent of solubilized liver hardly affects the anemia and leucopenia characteristic of animals on Ration S-44 alone but it does give, on the average, a definite improvement in growth. However, the growth obtained does not equal that of animals on stock ration. The missing substances appear to be supplied in part by alfalfa, grass juice powder, the cruder liver preparations, and, to a lesser extent, by other materials (corn steep powder, yeast).

The better growth obtained on Ration S-44 with the present series of animals is possibly due to intestinal synthesis. The pattern of growth of those animals which survive on Ration S-44, as well as the complete suppression of growth by succinylsulfathiazole, seem to favor this explanation. The success of Hogan (4) in raising guinea pigs on purified rations containing dextrin and cellulose, known to promote intestinal synthesis in the rat, seems to indicate the importance of this mechanism in the guinea pig.

How many dietary factors are required by the guinea pig is extremely uncertain. Animals receiving supplements of 25 per cent of linseed oil meal plus 4 per cent of solubilized liver (Ration S-45) grew better than on the basal ration with either supplement alone (Table I and Table III). Eight per cent of solubilized liver added to the basal diet (Table III) increases growth and renders the further enhancement by linseed oil meal not very impressive. The growth increase caused by the addition of solubilized liver to Ration S-44c is due to some factor other than "folic acid," which, however, Woolley and Sprince (3) claim is required by the guinea pig. Whole liver substance or liver 1:20 powder with Ration S-44c and grass juice powder, corn steep powder, yeast or alfalfa added to Ration S-45c produce growth in excess of that obtained by the addition of 8 per cent of solubilized liver to Ration S-44. (Animals on Ration S-44 grew no better with 8 per cent than with 4 per cent of solubilized liver added (1).) The data in Table I indicate that the growth promoting activity of these substances does not parallel their hemopoietic activity. The addition of succinylsulfathiazole brings this out more clearly (Table II).

This might be interpreted to mean that the guinea pig requires four substances or groups of substances in addition to "folic acid"; one in linseed oil meal, one in solubilized liver (besides "folic acid"), and two in crude liver preparations, grass juice powder, alfalfa, etc., one for growth and another for hemopoiesis. Much further work is

TABLE II

*Effect of Succinylsulfathiazole on Growth and Blood Status of Guinea Pigs Fed Natural Rations and Purified Rations With Various Supplements*

Ration	Growth		Blood			
			3 Weeks		6 Weeks	
	4 Weeks g. per day	6 Weeks g. per day	Leuco- cytes per mm <sup>3</sup>	Hemo- globin g. per 100 cc.	Leuco- cytes per mm <sup>3</sup>	Hemo- globin g. per 100 cc.
Ground Stock ration	7.1 6.4	6.3 7.6			5400 4200-5800	13.6 13.2-13.7
Ground Stock ration +1/2% succinyl-sulfathiazole	7.6 6.1	7.6 9.3	5400 3500-8100	12.6 11.1-13.4	4900 3100-6600	13.3 12.5-14.6
S-44c-ss (S-29c+25% linseed oil meal +1/2% succinylsulfathiazole)	0		1300	7.6		
S-44c-ss+4% solubilized liver (S-45c-ss)	3.0 0.3 5.4	1.4 3.8-4.6	1600 500-3700	9.2 8.3 10.4	3600 2200-3200	7.6 10.4-13.2
S-44c-ss+6% liver 1:20 powder	4.8 4.4-5.1	4.2 3.8-4.6	2400 1800-3000	9.6 8.8-10.8	2700 2200-3200	11.8 10.4-13.2
S-44c-ss+16% whole liver substance	4.5 3.6 5.4	5.1 4.2 5.8	3400 2300-1900	10.8 9.9 11.5	2700 1700 3200	11.3 11.0 11.7
S-45c-ss+16% alfalfa leaf meal	6.7 6.2-7.5	4.5 4.1-5.2	2000 1500-2300	10.7 10.6-10.8	1400 1100-1600	9.6 8.5-10.2
S-45c-ss+16% dried grass	5.7 4.8 6.4	3.5 1.5 6.4	2900 1800 3600	10.7 8.7 12.0	900 400 1800	9.7 9.6-9.9
S-45c-ss+16% soybean oil meal	4.4 2.0 5.7	5.8	1800 800-2800	10.1 9.5-10.3	3200 1	9.6 1
S-45c-ss+8% corn steep powder	4.8 3.9-6.3	3.5 2.5 4.5	2400 1400 4500	10.2 9.9 10.5	3600 2500 4800	10.7 10.4 10.9
S-45c-ss+33% ground stock ration	2.7 1.5 4.9	4.2 3.5 4.8	2600 1900 3500	10.4 10.1-11.0	4200 3000-5400	11.3 10.0-12.5

little improvement in survival, growth, or hemopoiesis, it would seem that at least one of the necessary factors is present in minimal quantity in the stock ration.

### DISCUSSION

The results presented here, while confirming much of the earlier work, emphasize the fact that solubilized liver does not supply all

TABLE I

*Substances Administered to Rats Receiving the Basal Diet Plus the Sulfonamide Drugs*

Group No.	Substance administered <sup>1</sup>	Rats numbers	Therapy days
Series A (1% Sulfaguanidine)			
1	"Vitamin" mixture <sup>2</sup>	15	10
2	Biotin <sup>3</sup> (1γ)	6	7
3	Xanthopterin (20γ) + biotin (5γ)	7	10
4	p-Aminobenzoic acid (3 mg.) + biotin (1γ)	7	14
5	Liver norit eluate (1 cc.) + biotin (1γ)	29	49
6	Wilson's whole liver substance (0.5 g.)	8	49
Series B (0.5% Sulfaguanidine)			
1	Liver norit eluate (1 cc.) + biotin (1γ)	10	42
2	Adenine-thiomethylpentoside (2 mg.) + biotin (2γ)	8	10
Series C (0.5% Sulfasuxidine)			
1	Biotin (2γ)	3	21
2	Adenine-thiomethylpentoside (2 mg.) + biotin (2γ)	3	10
3	Xanthopterin (20γ) + biotin (2γ)	4	7
4	p-Aminobenzoic acid (3 mg.) + biotin (2γ)	7	10
5	Liver norit eluate (1 cc.) + biotin (1γ)	3	5

<sup>1</sup> All substances were administered daily by subcutaneous injection, except the whole liver substance which was given orally. The values in parentheses indicate the daily dosages.

<sup>2</sup> The "vitamin" mixture furnished a daily dosage of 1 mg. each of nicotinic acid, adenine, and synthetic batyl alcohol; 2 mg. each of glutamine, pimeleic acid, uracil, guanine, xanthine, and yeast adenylic acid; 3 mg. of muscle adenosine triphosphate; and 5 mg. each of *t*-inositol and yeast nucleic acid.

<sup>3</sup> The biotin employed in the present studies was furnished as the S.M.A. concentrate No. 1000.

response was first observed in two other rats sixteen days after treatment with *p*-aminobenzoic acid plus biotin was discontinued. No effects were noted in the remaining 7 rats. Normal lymphocyte values were attained in only three animals receiving *p*-aminobenzoic acid plus biotin.

C. *Whole Liver and Norit Eluate-Biotin Therapies.* In animals fed 1% sulfaguanidine, the administration of norit eluate plus biotin resulted in a temporary growth response as indicated in Fig. 1 (Group 5, Series A). The growth-stimulating effect of whole liver was similar in character, the initial response being even less marked (Group 6, Series A). In contrast, treatment with norit eluate plus biotin in rats fed 0.5% sulfaguanidine led to an immediate and sustained growth response

(Group 1, Series B). A rate of growth comparable to the maximum rate noted in the control group was obtained during the six weeks of therapy (Fig. 1).

In animals receiving 1% sulfaguanidine, 0.5% sulfaguanidine or 0.5% sulfasuxidine, a pronounced rise in the number of granulocytes invariably occurred within two to five days after treatment with whole liver (Group 6, Series A) or norit eluate plus biotin (Group 5, Series A; Group 1, Series B; and Group 5, Series C) was begun (Fig. 2). With the continued administration of these substances, the granulocyte counts fell to a normal value and were maintained at that level during the period of therapy. The granulopoietic effects of whole liver and of norit eluate plus biotin were similar and are summarized together in Fig. 2. The increase in lymphocytes which resulted from these therapies was gradual in animals receiving 1% sulfaguanidine (Fig. 2). In contrast, norit eluate plus biotin therapy in animals receiving either 0.5% sulfaguanidine or sulfasuxidine resulted in an immediate lymphocyte response, values equal to those of the control rats being attained within five days (Fig. 2).

Treatment with either whole liver or norit eluate plus biotin for 2-5 weeks in rats fed 1% sulfaguanidine raised the hemoglobin content of the blood from a value of 10 to one of 14 g. per 100 cc.

## II. Therapeutic Activity of Methyl Acetamide (Series D)

Weanling rats were fed the basal diet with 0.5% sulfaguanidine for fifteen weeks prior to therapy. In addition to the usual vitamin B complex supplement, these animals received daily 3.0 mg. of *i*-inositol and 0.3 mg. of nicotinic acid in supplement dishes. The animals were weighed weekly and blood studies were performed biweekly.

After 12 weeks, the growth curve of the animals in this series plateaued at an average weight of 170 g. Leukopenia was noted after 15 weeks. At this time, crystalline biotin, methyl acetamide, and *p*-chloro-xylene were administered as indicated in Table II. The animals continued to receive the sulfonamide-containing diet during the period of therapy.

Although devoid of any growth-stimulating activity, methyl acetamide possessed leukopoietic ability as indicated in Table II. A consistent granulopoietic activity of methyl acetamide was noted at daily doses of 40 and 80 mg., the higher dose having a more pronounced effect. Occasional granulopoietic responses were elicited by a daily dose

TABLE II  
*Leukopoietic Activity of Methyl Acetamide and p-Chloro-Xylenol  
in Rats Receiving 0.5% Sulfguanidine<sup>1</sup>*

Therapy <sup>2</sup> Type	Therapy days	Rats number	Granulocytes <sup>3</sup>		Lymphocytes <sup>3</sup>	
			Before therapy	After therapy	Before therapy	After therapy
30 mg. P.C.X. plus 80 mg. M.A.A.	5	9	190 (80-300)	2800 (1000-4700)	5600 (4000-6100)	7900 (5000-14000)
80 mg. M.A.A.	5	9	270 (100-500)	2700 (1200-4900)	5200 (2500-6500)	8500 (5100-15000)
40 mg. M.A.A.	8	4	410 (325-500)	1600 (1200-2000)	5100 (3500-6500)	7800 (5000-10000)
10 mg. M.A.A.	6	9	180 (25-300)	820 (200-1400)	4900 (3300-6600)	6100 (2300-12000)

<sup>1</sup> These compounds were administered daily by subcutaneous injection to rats of Series D. Each rat received 2 γ of crystalline biotin orally during the period of therapy.

<sup>2</sup> P.C.X. and M.A.A. refer to *p*-chloro-xylenol and methyl acetamide, respectively. P.C.X. was dissolved in M.A.A. when these compounds were given together. M.A.A. was dissolved in isotonic saline when given separately.

<sup>3</sup> Average values are expressed as cells per cmm. of blood. Range of values is given in parentheses.

of 10 mg. of methyl acetamide. *p*-Chloro-xylenol had no supplementary activity when given with 80 mg. of methyl acetamide. The lymphocyte response to methyl acetamide was not marked.

Microscopic examination of sections of sternal bone marrow from animals autopsied immediately after the cessation of methyl acetamide therapy showed a high positive correlation between the hematopoietic activity of the marrow and the number of circulating leukocytes. Poor hematopoietic activity was noted in marrows from animals in this series which did not receive methyl acetamide.

### III. Mortality and Symptomatology

The appearance of the control rats was normal in all respects and no deaths occurred in this group during the course of the experiment.

The symptoms observed in the animals receiving the basal diet with 1% sulfaguanidine, 0.5% sulfaguanidine or 0.5% sulfasuxidine (Series A, B, C, and D) were very similar in character and generally appeared within 9 weeks. The condition of the animals became progressively worse and 85% of the rats in Series A, B, and C, not receiving liver or liver norit eluate, died within 15 weeks on experiment. A longer survival time was noted in animals receiving *i*-inositol and nicotinic acid (Series D). Animals that received liver or liver norit eluate survived until the end of the experiment, except for a small percentage that died within a few days after treatment was begun.

Approximately 15% of the animals exhibited a ventral alopecia and the condition generally described as "spectacle eye." Spontaneous remissions of both of these symptoms occurred frequently, thus making difficult any attempt to evaluate a specific therapy. However, neither symptom was present in rats receiving either liver or liver norit eluate-biotin therapies.

Cheilosis was observed in 25% of the rats and was characterized by transverse fissures at the corners of the mouth and a shiny, red, edematous appearance of the lower lip. Both liver and liver norit eluate-biotin therapies were effective in preventing or curing this condition.

Spasticity of the hind legs which resulted in a characteristic "hopping" gait was encountered frequently in the animals. This symptom could be correlated with the general condition of the animal, being most pronounced when the rat was in a debilitated state. Cures always resulted from the administration of liver, or liver norit eluate plus biotin. Treatment with biotin alone was ineffective.

## DISCUSSION

The mechanism through which sulfaguanidine or sulfasuxidine exert their toxic effects in rats fed purified diets is not completely understood. Available evidence favors the view that they function by virtue of their bacteriostatic action upon various types of intestinal bacteria which synthesize certain substances required by the host. In accordance with this hypothesis, these sulfonamides induce a vitamin deficiency which, in our experiments, is curable by substance(s) present in the norit eluate fraction of liver when given with biotin concentrate SMA No. 1000. These substances are normally furnished through the synthetic activities of the intestinal flora. The norit eluate fraction of liver contains a variety of biologically active factors (variously desig-

nated as *L. casei* factor, vitamin B<sub>e</sub>, folic acid, and vitamins B<sub>10</sub> and B<sub>11</sub>) and Daft and Sebrell (9) have reported the anti-sulfonamide activities of related crystalline compounds under conditions similar to those reported in the present paper. This subject has been adequately reviewed elsewhere (10).

It is apparent that the toxicity of 1% sulfaguanidine is not completely counteracted by dietary means. This is evidenced by the inability of whole liver, or norit eluate plus biotin to restore normal growth and by the delayed lymphocyte response to these therapies. In contrast, norit eluate-biotin therapy produces immediate and sustained growth and lymphopoietic responses in animals fed 0.5% sulfaguanidine. Preliminary experiments indicate that thyroxin has no growth-stimulating activity when fed to rats receiving 1% sulfaguanidine in diets containing 10% of whole liver. Treatment with thyroxin was instituted in view of the belief that the administration of sulfaguanidine results in an impairment of thyroid hormone synthesis which is not counteracted by various dietary factors (11, 12).

The pronounced leukopoietic effect of methyl acetamide in human subjects has been demonstrated by Zondek and Bromberg (13). Although inactive by itself, *p*-chloro-xyleneol was found to enhance the effect of methyl acetamide. Evidence was presented by these authors to support the assumption that methyl acetamide plus *p*-chloro-xyleneol stimulated the granulopoietic function of the bone marrow. In our experiments, methyl acetamide was found to have leukopoietic activity in the leukopenia produced by the administration of 0.5% sulfaguanidine. Histological studies indicated a direct stimulation of the hematopoietic activity of the bone marrow by methyl acetamide. A possible metabolic inter-relationship between methyl acetamide and the active factor(s) in the liver norit eluate suggests itself. Whether methyl acetamide is converted by the rat into the hematopoietic factor(s) or in some fashion influences the metabolism of this factor(s) is a matter of speculation. It is to be noted that methyl acetamide was devoid of any growth-stimulating action, and that the amount of methyl acetamide required for leukopoiesis was far greater than that of the vitamin B<sub>e</sub> equivalents present in a dosage of liver norit eluate sufficient for marked leukopoiesis.

Attempts to demonstrate a relationship in bacteria between methyl acetamide and factors in the norit eluate gave negative results. Thus, methyl acetamide at levels from 0.0005 γ to 200 γ per 10 cc. of medium,

both in the absence of the vitamin B<sub>c</sub> reference standard (Parke, Davis and Co.) and in the presence of suboptimal amounts of this concentrate, had no influence upon the growth of either *L. casei* or *Strep. faecalis R* under conditions where certain factors in the norit eluate are limiting for growth (5, 14).

### SUMMARY

The detrimental effects of sulfaguanidine and sulfasuxidine upon growth, appearance, and hematopoiesis in rats fed a purified diet have been described. A variety of substances have been tested for their therapeutic effectiveness with the following results:

1. Nicotinic acid, adenine, synthetic batyl alcohol, glutamine, pimelic acid, uracil, guanine, xanthine, yeast adenylic acid, muscle adenosine triphosphate, *i*-inositol, yeast nucleic acid, biotin, xanthopterin and adenine-thiomethylpentoside were ineffective.
2. *p*-Aminobenzoic acid plus biotin had no growth-stimulating effect in rats fed 1% sulfaguanidine. In some instances, this therapy partially counteracted the growth inhibition in rats receiving 0.5% sulfasuxidine. The administration of *p*-aminobenzoic acid plus biotin resulted in a variable and delayed leukocyte response in rats receiving either 1% sulfaguanidine or 0.5% sulfasuxidine.
3. Marked growth responses resulted from the administration of the liver norit eluate fraction plus biotin to rats receiving 0.5% sulfaguanidine. In contrast, liver norit eluate plus biotin, or whole liver was only partially effective for growth in rats receiving 1% sulfaguanidine. The leukopenia in animals receiving the sulfonamides could be cured with either whole liver or the liver norit eluate fraction plus biotin.
4. Methyl acetamide possessed leukopoietic potency attributable to its direct action on the bone marrow in the leukopenia resulting from the administration of 0.5% sulfaguanidine. Experiments with *L. casei* and *Strep. faecalis R* gave no evidence for a metabolic relationship between methyl acetamide and factors in the liver norit eluate fraction.

### ACKNOWLEDGMENTS

We wish to express our indebtedness to the Wilson Laboratories for the liver Extract L; to Merck and Co., Inc., for the B vitamins and the 2-methyl-1,4-naphthoquinone; to Parke, Davis and Co. for the reference vitamin B<sub>c</sub> standard; to the Lederle Laboratories, Inc., for the sulfaguanidine; to Sharp and Dohme, Inc., for

the sulfasuxidine; to Dr. W. A. Lott of the Squibb Institute for Medical Research for a sample of xanthopterin; to Dr. Harry N. Holmes for the synthetic batyl alcohol; and to Dr. F. Schlenk for the adenine-thiomethylpentoside.

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# Oxygen Production by Anaerobic Photosynthesis of Algae Measured by a New Micromethod

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Received February 23, 1945

## PART ONE—THE EXPERIMENTS

### *Introduction*

Recently (1), a method has been described by which the production of very small quantities of oxygen can be measured in gases and solutions which are originally free of oxygen. Preliminary measurements have shown that the sensitivity of this method is more than sufficient to follow the oxygen production by an irradiated suspension of isolated chloroplasts. However, the practically complete absence of oxygen which is essential for the application of the new method, introduces certain problems which had best be studied first with normal intact organisms. The present paper describes such studies with the algae, *Chlorella* and *Scenedesmus*.

### *Method and Typical Results*

We shall outline the method briefly, emphasizing those features which influence the interpretation of the results. Small quantities of oxygen strongly quench the phosphorescence of the dye, trypaflavine, adsorbed on silica gel (2). By measuring the phosphorescence intensity while a stream of nitrogen passes very slowly over such a phosphor, changes of oxygen content in the gas can be observed. The oxygen pressure can be calculated with the aid of a calibration curve obtained with gas mixtures containing known proportions of oxygen. The

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\*\* National Research Council Fellow, 1943–44.

phosphorescence intensity was measured with a phototube of the electron multiplier type connected with a galvanometer. In our experiments, curves were constructed from galvanometer readings taken every two seconds. From these curves one obtains the number of oxygen molecules produced per minute by means of the relation

$$n = \frac{\bar{p}}{760} uN$$

where  $\bar{p}$  is the average oxygen pressure during the one minute period,  $N$  Loschmidt's number, and  $u$  the rate of gas flow in cc. per min.

To observe the rate of oxygen production by algae, pure nitrogen (containing less than one part oxygen in  $10^6$ ) is bubbled through a cell containing the algae suspended in 2.5 cc. of water. (In some cases, hydrogen of the same purity was used instead of nitrogen.) If, on illumination of the algae, oxygen is produced, it is carried away by the gas stream which flows over the phosphor and the intensity of phosphorescence shows a decline.

Cultures of *Scenedesmus* and *Chlorella* were grown as usual in this laboratory. Before being used for an experiment, the algae were exposed to strong illumination for two hours in a continuous current of air containing 4% of carbon dioxide in a constantly agitated vessel. A sample was withdrawn and the concentration of the algae was measured in a Klett colorimeter, after which it was diluted with water or concentrated by centrifugation in order to bring it to the desired level ( $10^{-6}$  to  $10^{-3}$  g. of algae per cc.).

$2\frac{1}{2}$  cc. of this suspension were introduced into the vessel sketched in Fig. 1.  $O$  is a filling tube which can be closed with a ground stopper.  $F$  is a filter of fritted glass through which a constant current of pure nitrogen is forced under pressure in the direction of the arrows; because of this pressure most of the liquid is held in the volume above  $F$ , where it is continually flushed by the small bubbles of nitrogen. The vessel is enclosed in a double-walled metal box,  $B$ , which can be filled with water of desired temperature and serves both as a thermostat and as a screen against stray light from outside.  $T$  is a copper-constantan thermocouple. A cylindrical opening through the side wall of the box provides an entrance for the light source,  $L$ . A glass window seals the inner end of the cylinder and the lamp support slides into the outer end so that the bulbs can be easily exchanged in the dark. A hot filament bulb of the type Mazda 1183, 6 V, 50 c.p. serves for constant illumination. Weaker light intensities needed for the measurement of saturation curves were obtained by placing the lamp outside the box, with an appropriate system of lenses and screens to provide the desired illumination. Mazda No. 5 photoflash bulbs were used for flash experiments.

During an experiment the room was kept dark except for the excitation lamp for the phosphor and a small reading lamp. The nitrogen was practically free of carbon dioxide; titration showed the partial pressure of carbon dioxide to be less than  $10^{-6}$  mm. For experiments in which a constant supply of carbon dioxide was needed, a vessel similar in structure to that containing the algae was filled with half molar

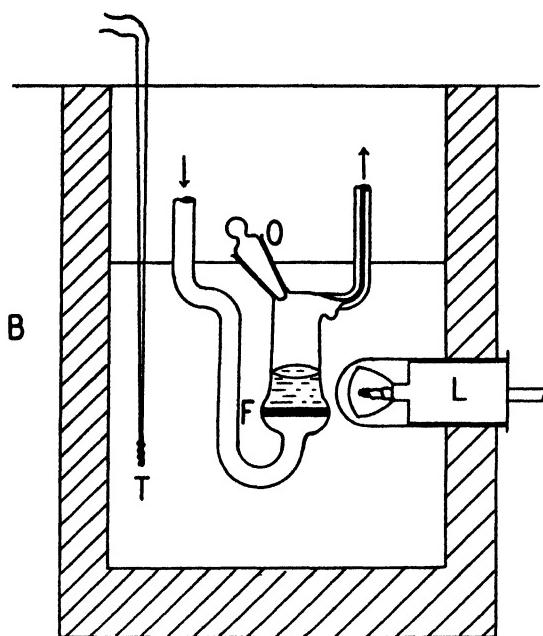


FIG. 1  
Diagram

potassium bicarbonate and inserted in the gas supply line. The nitrogen, or hydrogen, after having bubbled through this solution was found to contain 2% of carbon dioxide. Both vessels had to be flushed with oxygen-free gas for at least three hours before the beginning of an experiment to eliminate all traces of oxygen in the system and to insure the full intensity of phosphorescence. During the experiments the gas current was maintained at a rate of approximately three cc. per minute.

There are some features common to all quenching curves which are characteristic of the method rather than of the true rate of oxygen liberation. The liberated oxygen first passes into the water, from which it must be swept out by nitrogen or hydrogen and then travels through a length of capillary tubing before reaching the phosphor. The last mentioned fact merely causes a time lag between the beginning of oxygen development and its observation, but the first one leads to certain distortions of the rate curves. An instantaneous change of the true rate produces only a gradual change of the observed intensity of phosphorescence. For example, an outburst of oxygen production

lasting about 0.01 second gives a depression in the light intensity curve of the phosphor which has the shape of a Gaussian curve with a half width of about ten seconds. The slopes of the curves at the beginning and end of illumination are similarly flattened. This effect was found to be particularly marked at the end of illumination periods if the oxygen production was strong and of long duration. Apparently, some oxygen becomes adsorbed on the glass, especially on the fritted glass filter, and is only slowly removed by the stream of pure nitrogen. Quenching curves obtained with oxygen produced by electrolysis in the same reaction vessel showed similar characteristics, proving that the delay was not caused by a slow gas exchange between the algae and solution.

Special care has to be taken to keep the fritted glass clean. If, after an experiment, the algae are removed from the vessel by means of chromic acid, finely divided carbon is likely to be left in the pores of the filter. This carbon adsorbs oxygen from the air and the latter is only very slowly desorbed by the stream of pure nitrogen, so that even after four to five hours of flushing with oxygen-free gas the latter remains contaminated, and the phosphorescence is quenched even when the algae are in the dark. Cleaning the apparatus by boiling in concentrated nitric acid or by prolonged heating in an annealing oven removes this carbon residue efficiently.

Figures 2a, b, 3a, b, and 4a, b show the types of curves which we have observed. Fig. 2a shows the successive galvanometer readings obtained with an illuminated suspension of *Chlorella*. The start of the illumination period is marked by an arrow pointing upward; its end by a downward pointing arrow. Curve 2b is a corresponding plot of the partial pressure of oxygen in the gas passing over the phosphor, obtained from Curve a with the help of the calibration curve. The slope of the calibration curve is small at the higher oxygen pressures, so that above a partial pressure of  $1.5 \times 10^{-3}$  mm. Hg the measurements become very inaccurate. Even at lower pressures the absolute values can claim only an accuracy of  $\sim \pm 100\%$ , since it was too time-consuming to repeat the calibration before each experiment. If the behavior of the phosphor indicated that its sensitivity had changed, the phosphor was reactivated or replaced and a new calibration curve was determined.

Curves 3 a, b and 4 a, b are corresponding examples of curves observed with *Scenedesmus*. The curves show a time lag of about 20 seconds between the time the light is turned on and the arrival of the first oxygen-contaminated gas at the phosphor. The same

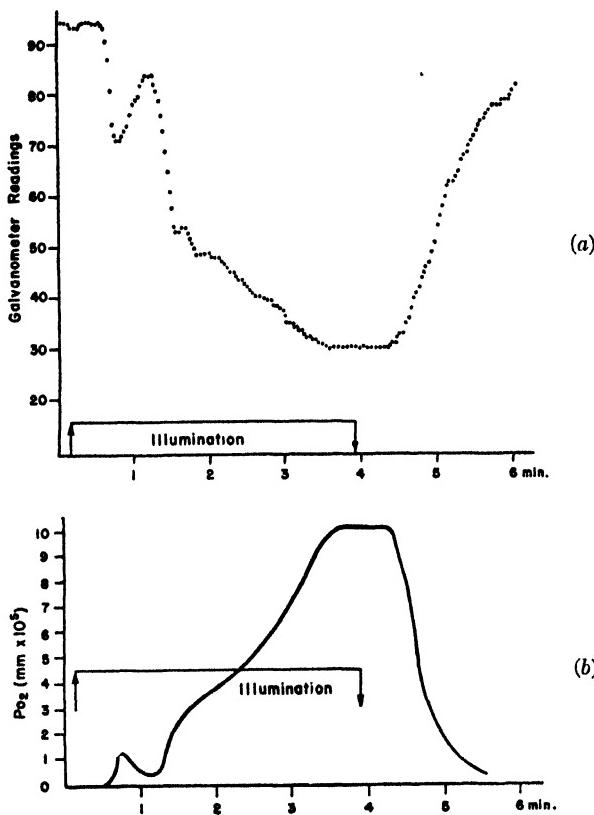


FIG. 2

(a) Galvanometer Time Curve during Illumination of  $7.5 \times 10^{-4}$  g. *Chlorella* in  $2\frac{1}{2}$  cc. Solution after Three Hours Anaerobicity in Nitrogen  
 (b) Corresponding Time Curve of Oxygen Production

time lag is observed between the end of the illumination period and the first decline of the oxygen concentration at the phosphor. All three sets of curves were measured in nitrogen at room temperature in presence of about 20 mm. of carbon dioxide. The light intensity was sufficient to obtain the light saturation of photosynthesis. A discussion of the variations in the shape of the curves and differences in the maximum rate of oxygen production will be found in the following sections. Curves 5, I and II, show results

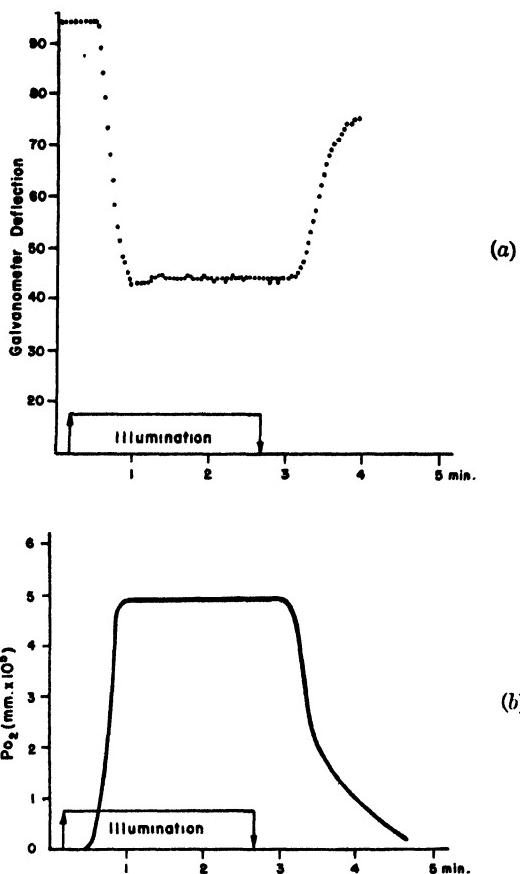


FIG. 3

- (a) Galvanometer Time Curve during Illumination of  $5 \times 10^{-3}$  g. *Scenedesmus* in  $\frac{1}{2}$  cc. Solution after Three Hours Anaerobicity in Nitrogen  
 (b) Corresponding Time Curve of Oxygen Production

typical for the oxygen production by single light flashes with the algae *Chlorella* and *Scenedesmus*. Each flash, with exception of the second,<sup>1</sup> was produced by igniting a No. 5 photoflash bulb at 1 cm.

<sup>1</sup> The double arrow in Fig. 5 a indicates two seconds of illumination with light of intensity that would produce saturation with continuous irradiation. The comparison shows the much greater efficiency of the flash illumination.

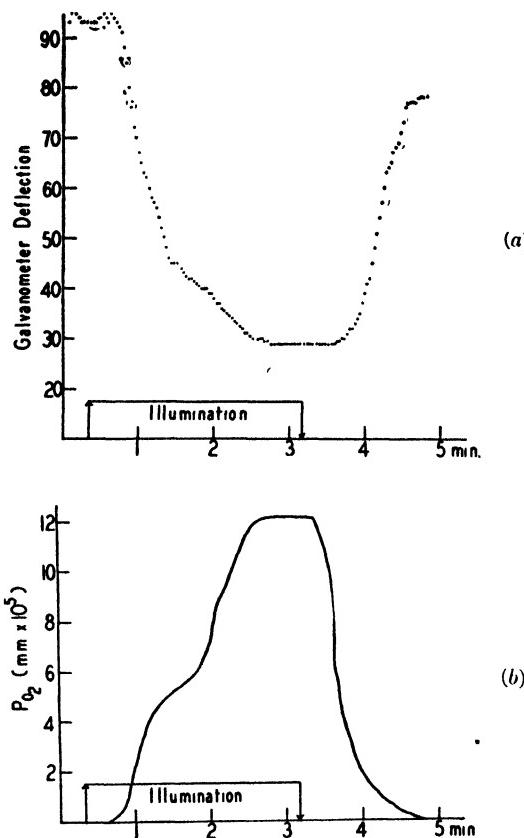


FIG. 4

(a) Galvanometer Time Curve during Illumination of  $5 \times 10^{-3}$  g. *Scenedesmus* in 2½ cc. Solution after Three Hours Anaerobicity in Nitrogen  
 (b) Corresponding Time Curve of Oxygen Production

distance from the vessel. Curve 6 shows the light intensity as a function of time for an ignited photobulb. The flash lasts for more than 0.01 second. The illumination period is thus longer than the Emerson and Arnold (3) period at room temperature (working period of *cat B* according to the theory of Franck and Herzfeld, 14); at 0°, however, the Emerson-Arnold period is considerably longer and the flash illumination is short by comparison with it.

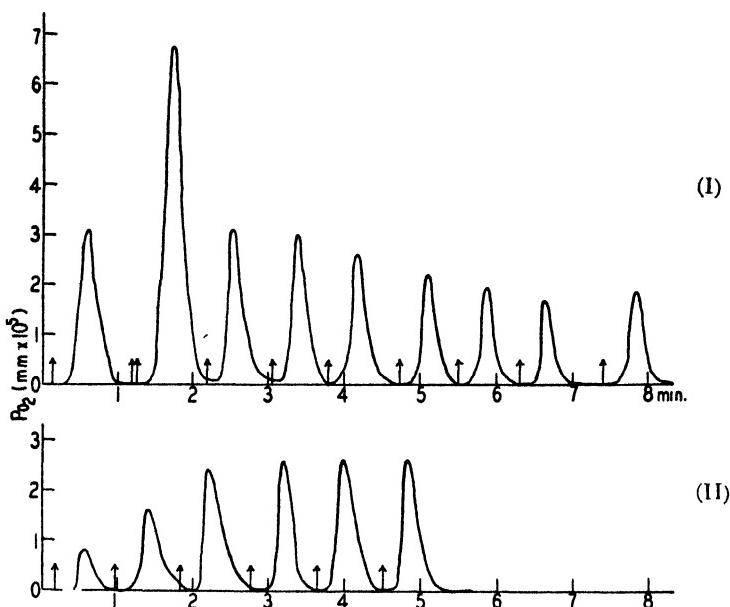


FIG. 5

(I) Oxygen Production by Light Flashes. *Chlorella*(II) Oxygen Production by Light Flashes. *Scenedesmus*

(For explanation of double arrow, see Footnote 1.)

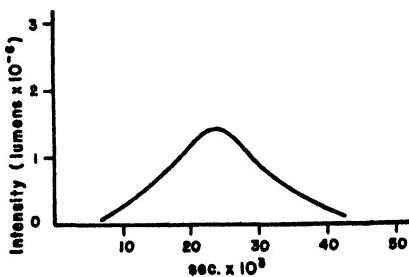


FIG. 6

Light Intensity as a Function of Time for an Ignited Photoflash Bulb  
(Reprinted from *Photopedia*, 1940-41.)

### I. Saturation Curves in Nitrogen and Hydrogen

Light saturation curves have been measured with *Chlorella* and *Scenedesmus* under a variety of conditions in atmospheres of nitrogen and of hydrogen. For each point on these curves the oxygen liberation was measured long enough to ascertain that a constant rate had been reached.

The shape of the saturation curves, as well as the saturation rates, differ from the usual ones, observed under aerobic conditions. Fig. 7, I and II, gives examples of the two types of curves observed. Curve I

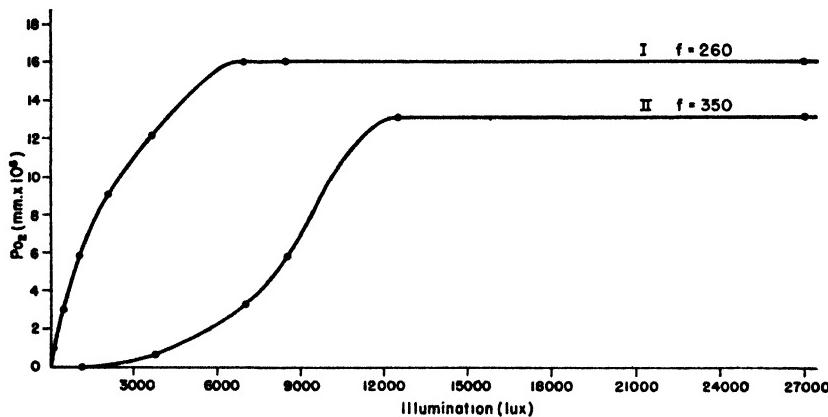


FIG. 7

Light Saturation Curves for Oxygen Production by  $12.5 \times 10^{-4}$  g. Algae in  $2\frac{1}{2}$  cc. Solution after Three Hours Anaerobicity in Nitrogen  
( $f$  designates reduction factor from normal aerobic saturation rate.)

is similar to normal saturation curves in shape, but the saturation rate is many times smaller than in air and the light intensity at which saturation is reached is much lower than under aerobic conditions. In the particular example represented by Fig. 7, I, saturation was reached at  $\sim 6800$  lux, while under otherwise similar aerobic conditions it was reached only at  $\sim 17,000$  lux; the maximum oxygen liberation in Fig. 7, I, was only  $1/260$  of that which the same amount of algae shows under aerobic conditions.<sup>2</sup> Curves of this type resemble saturation curves measured aerobically in the presence of poisons, e.g.,

<sup>2</sup> In the figures a reduction factor of, e.g., 260, is designated as  $f=260$ . The significance of these numbers will be discussed.

cyanide, if the latter are present in a concentration high enough to impose extreme enzymatic limitations. It is observed regularly in nitrogen as well as in hydrogen atmosphere, after about three hours of anaerobic conditioning, with *Chlorella*, as well as with *Scenedesmus*.

Occasionally one observes under the same conditions curves of a sigmoid shape. Curve 7, II, may serve as an example. Saturation is reached in this particular case at about 11,000 lux and the saturation yield is 350 times smaller than in air. Thus, the development of the sigmoid shape of the curve is linked to a shift of the saturation intensity

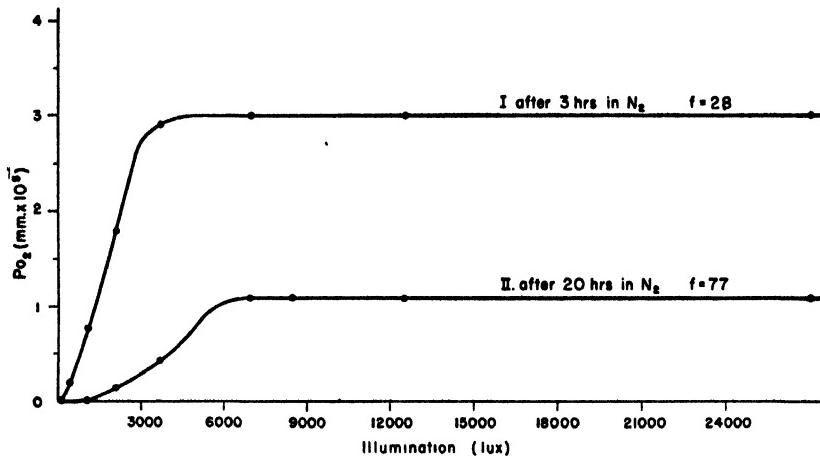


FIG. 8

Light Saturation Curves for Oxygen Production by  $2.5 \times 10^{-5}$  g.  
*Chlorella* in  $2\frac{1}{2}$  cc. Solution

( $f$  designates reduction factor from normal aerobic saturation rate.)

to higher values and a renewed decrease in the saturation rate. The upper limit to which the saturation intensity has been observed to shift was equal to the saturation intensity in air. Under the conditions when sigmoid saturation curves are obtained the time course of oxygen production becomes somewhat abnormal; the steady state is reached less rapidly than usual, indicating an induction period (as in Fig. 4). We also observed a correlation between the sigmoid type of saturation curves and unusually small yields of oxygen produced by single light flashes. (For more details, compare Sections II and III.) If the anaerobic treatment is prolonged to ten or twenty hours, the

transition to *Type II* becomes more pronounced, and the saturation rate grows systematically smaller. Curves 8, I and II, give as an example the curves obtained with *Chlorella* in nitrogen and carbon dioxide after three hours and twenty hours, respectively. Note that the saturation rate goes down after prolonged exposure to anaerobic conditions, while the saturation intensity rises; at the same time the shape of the curve at low intensities becomes sigmoid. At the beginning of an experiment it is not possible to predict whether a *Type I* or *Type II* saturation curve will be observed. The change from one type to the other depends, similarly to the induction phenomena, upon the age of the culture and upon small changes in the plant metabolism which we did not try to control.

It is remarkable that with the method described no systematic differences are observable between saturation curves measured in nitrogen and in hydrogen, nor between *Scenedesmus* and *Chlorella*. Both types occur in both gases and with both species of algae; the saturation rates of oxygen production are of the same order of magnitude in both, and the dependence upon the duration of the anaerobic conditions is similar in all cases. We had expected that *Scenedesmus* would produce no oxygen at all in an hydrogen atmosphere as long as the conditions were suitable for photoreduction. Gaffron (4) found that after three to twenty hours conditioning in hydrogen *Scenedesmus* (but not *Chlorella*) begins to take up two hydrogen molecules for each molecule of carbon dioxide reduced and that no oxygen development is observable with the Warburg manometer below a certain limit of light intensity. To find out whether this discrepancy was caused by the differences between the two methods of measurement employed or by different treatment of the algae, two sets of experiments were made, in which Dr. Gaffron cooperated. A sample of algae was divided in two parts, the one was used for manometric measurements and the other for measurements by the phosphorescence method. With the manometer the algae showed the phenomenon of photoreduction in a hydrogen atmosphere as usual, while with the phosphorescence method the results were as described above. A set of measurements was then made with the phosphorescence method but with a concentration of algae as high as that used for the manometric method. The result is shown by Fig. 9. In general, the curves in Fig. 9 are not different from those obtained with the lower concentration of algae under the same conditions; that the sigmoid type curve is already

developed after four hours of anaerobiosis is not unusual, but the tendency to adopt this type of saturation curve seems to be a little more pronounced in hydrogen than in nitrogen.

Further experiments have been made to uncover the mechanism by which the lack of oxygen reduces the saturation yield. Most of the measurements were made after three hours in a nitrogen atmosphere. Under these conditions one usually has to deal with only one type of saturation curve represented by Curve 7, I. One external factor was changed at a time, while the others were kept as constant as possible. In cases in which small changes in the age of the culture and previous

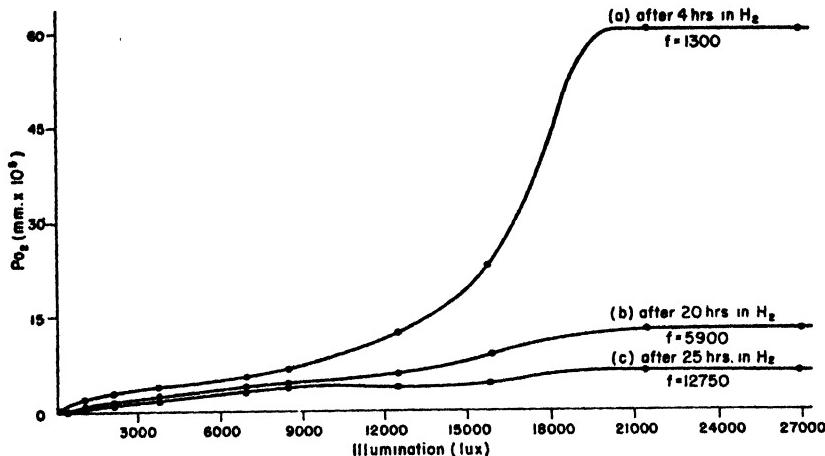


FIG. 9  
Light Saturation Curves for Oxygen Production by  $22 \times 10^{-3}$  g.  
*Scenedesmus* in 2½ cc. solution

treatment could be decisive, the comparison was made with two observation vessels arranged parallel to each other so that all the conditions were identical except the one under investigation. That procedure was used, for instance, to find out whether the pH of the solution in which the algae are suspended has an influence on the time course of photosynthesis or on the saturation rates.

Aufdemgarten (5) observed that under aerobic conditions changes in the induction phenomena occurred if the algae were grown in an alkaline solution instead of the slightly acid solution normally used. Noack and co-workers (6) reported that *Chlorella* which had been kept for ten to twenty hours in the dark in an alkaline solution

started to photosynthesize without a prolonged induction period. If, on the other hand, the solution was slightly acid, no photosynthetic activity could be detected in their manometers, even after hours of strong irradiation. Our own observations did not reveal significant differences either in the time course or in the saturation rate between algae which were kept for three to five hours in suspensions of pH 6 or of pH 9. The possibility that minor changes may occur after prolonged dark times or with algae grown in solutions of different pH is not excluded by the present measurements. It will be shown in the discussion that very small changes may be responsible for Noack's results.

Experiments in which the temperature was varied between 25°C. and 0°C. revealed that when the saturation yield is strongly reduced by absence of oxygen the temperature coefficient of photosynthesis is much smaller than under normal conditions. In air the saturation rate for *Scenedesmus* measured with the Warburg manometers<sup>3</sup> is about twenty times greater at 20°C. than at 0°C. Our observations gave different temperature coefficients, depending on the concentration of algae chosen. In one case, the saturation rate of oxygen production dropped at 0°C. to only one-half of its value at 20°C. (the rate at room temperature in this case was ~ 250 times smaller than the rate measured aerobically); in another experiment with a much more dilute algal concentration the oxygen yield at 20° was only ten times smaller than the aerobic yield (cf. below on the effect of concentration on yield), and the saturation rate dropped to one-fifth if the temperature was lowered from 20° to 0°C. The sensitivity against cyanide is similarly lowered by anaerobic conditions. Cyanide concentrations sufficient to depress the photosynthetic rate in air to a few per cent reduce the rate only by a small factor, if it has already been drastically lowered by the anaerobic conditions. As cyanide is known to limit the carbon dioxide uptake, its influence has analogies to carbon dioxide limitations imposed by a low partial pressure of this gas. It was, therefore, expected that the influence of extreme reduction of the carbon dioxide content in the nitrogen would have a much smaller effect than under aerobic conditions. This expectation was tested by comparison of the saturation production of oxygen in an atmosphere of nitrogen plus 20 mm. of carbon dioxide with the one in pure nitrogen. This nitrogen contained less than  $10^{-5}$  mm. of carbon dioxide, before entering the vessel with the algae; but some carbon dioxide is always formed by the fermenting algae. We found that the partial pressure developed in that way was  $5 \times 10^{-4}$  mm., with a concentration of

<sup>3</sup> Dr. Gaffron kindly made these measurements for us.

$12.5 \times 10^{-3}$  g. of algae in 2.5 cc. of solution at room temperature. It is, therefore, about 2000 times smaller (7) than the partial pressure needed to avoid carbon dioxide limitations under aerobic conditions. The oxygen production in air under this extreme carbon dioxide limitation would be lowered by a factor of about 1000. Under anaerobic conditions the oxygen production in presence of carbon dioxide was only ten times higher than that without carbon dioxide addition. The conditions chosen in the particular experiment mentioned were such that in nitrogen plus carbon dioxide the oxygen yield was eight hundred times smaller than in air. If pure nitrogen without admixture of carbon dioxide was used, the concentration of carbon dioxide present depends upon the rate of fermentation. Since its rate is known to rise with temperature and with the sugar content of the algae the saturation rate of oxygen production is expected to rise if the temperature is raised or glucose is added. That was confirmed experimentally.

The main factor controlling the yield of oxygen under anaerobic conditions turned out to be the concentration of the algae: *Ceteris paribus*, the higher the concentration the lower the yield per unit weight of algae. The great sensitivity of the method permits the use of algal suspensions which are so diluted that to the eye they are indistinguishable from pure water. No trace of green color is visible even by looking through columns of suspension about 10 cm. deep whose concentration is  $1 \times 10^{-5}$  g. of wet algae per cc. Nevertheless, such a suspension is in many respects the most suitable object for measuring saturation curves by our method. (The concentration usually chosen for manometric measurements is about  $5 \times 10^{-3}$  g. per cc.) With these extremely small concentrations of algae, the yield of oxygen at room temperature after  $\sim$  three hours of anaerobic treatment is only about ten times smaller than in air. At 0°C. the yield under anaerobic conditions is as much as one-half of that in air. Table I gives the results of measurements with concentrations varying from  $1 \times 10^{-5}$  g. per cc. to  $2.5 \times 10^{-3}$  g. per cc., taken with separate samples of the same culture of *Scenedesmus* at 20° and 0° after three hours of anaerobic treatment. All other external conditions have been kept as equal as possible.

For the comparison of anaerobic yields with the yield of photosynthesis in air, Gaffron's manometric measurements are quoted at the end of the table.

There is no fundamental difference between the concentration effects in *Scenedesmus* and *Chlorella*, nor between the effects in nitrogen and in hydrogen atmospheres. In order to make certain that changes of concentration influence the photosynthetic production of oxygen and that their effect is not caused by a subsequent loss of oxygen, experiments were made to find out whether respiration of the algae or photoxidation could produce a considerable loss of oxygen under the prevailing conditions. The influence of respiration was studied by allowing the stream of nitrogen to pass through two vessels in

TABLE I  
*Measurements Carried Out with Scenedesmus*

	Concen- tra- tion (g wet algae per cc solution)	Relative concen- tra- tion	Oxygen pressure during illumination	Relative oxygen production	Volume at 1 atmosphere of oxygen produced in 10 minutes	Yield of oxygen per g wet algae in 10 minutes	Reduction factor from normal rate
20°C	$1 \times 10^{-6}$	1	$0.5 \times 10^{-4}$	1	$0.0020$	80	17*
	$5 \times 10^{-6}$	5	$4.5 \times 10^{-4}$	9	$0.0177$	142	9
	$5 \times 10^{-4}$	50	$5.6 \times 10^{-4}$	11	$0.0221$	18	74
	$2.5 \times 10^{-3}$	250	$8.9 \times 10^{-4}$	18	$0.0351$	6	223
0°C	$1 \times 10^{-6}$	1	$0.1 \times 10^{-1}$	1	$0.0004$	16	4*
	$5 \times 10^{-6}$	5	$1.0 \times 10^{-4}$	10	$0.0039$	31	2
	$5 \times 10^{-4}$	50	$2.2 \times 10^{-4}$	22	$0.0087$	7	10
	$2.5 \times 10^{-3}$	250	$4.4 \times 10^{-4}$	44	$0.0173$	3	22

The yield of oxygen per g wet algae in air is 1340 mm.<sup>3</sup> in 10 minutes at 20°C.; 67 mm.<sup>3</sup> in 10 minutes at 0°C (Gaffron).

\* The fact that these particular values are larger than expected will be discussed.

series. In the first vessel suitable amounts of oxygen were produced photosynthetically and admixed to the nitrogen. This gas mixture then passed either directly into the phosphor tube or through the second vessel, which was kept in the dark and which contained algae in high concentration. If considerable losses of oxygen by respiration had occurred, the quenching will be much greater in the first case than in the second one. No difference was found, however, proving that the loss of oxygen by respiration is negligible. If a loss of oxygen does occur by photoxidation, the effect should increase with the light intensity. In that case the saturation curve would not have a plateau;

rather, the rates would rise to a maximum and then decline again. In our experiments the saturation rate remained constant, even in the region of very high light intensities. That excludes photoxidation as an explanation of the concentration effect.

From these results, we are forced to conclude that the photosynthetic production of oxygen itself is limited by the absence of oxygen. As mentioned above (page 111), the shape of the saturation curve after three to five hours of anaerobic treatment indicates that the limitation is caused by poisoning of an enzyme involved in one of the dark reactions of photosynthesis. There is no reason to doubt that the level of the poison will rise by prolongation of the anaerobic period, and this would easily explain a further reduction of the oxygen production with time. But apparently there is also a second reason for this fall of the oxygen yield with an increase of anaerobic treatment from three to twenty hours. This is indicated by the transition of the saturation curve of *Type I* into that of *Type II*. It was mentioned that even after only three hours saturation curves of *Type II* sometimes occur, together with low saturation rates, induction losses, and other characteristics associated with this type of curves. That was obviously the case in Table I in the experiment with  $1 \times 10^{-5}$  g. per cc. of *Scenedesmus* in nitrogen, which shows a smaller output than expected for the low concentration. The saturation curve itself was not measured in that case, but the time course shows clearly the induction loss, a definite indication that the saturation curve belonged to *Type II*. The sigmoid shape of *Type II* indicates a narcotic limitation, as will be pointed out in detail in the discussion in Part Two.

## II. Transient Phenomena in Oxygen Production

A. *Induction Phenomena.* Examples of the different shape of the time curves of oxygen production for *Chlorella* and *Scenedesmus* have been given in the Figs. 2, 3, and 4. The curve of Fig. 3 is the one most frequently observed with *Scenedesmus* after three to five hours of anaerobicity in atmospheres of nitrogen or hydrogen with an admixture of ~ twenty mm. of carbon dioxide at high or at low temperatures. No induction phenomena are observable. The oxygen production rises to its final rate as rapidly as the inertia of the method permits, so that if induction occurs it must last for less than about twenty seconds. (Experiments with light flashes and observations

of the time course of fluorescence presented below indicate the presence of such brief induction phenomena at the very beginning of the irradiation.)

Occasionally after three hours of anaerobic treatment, but much more often after longer anaerobic treatment, curves of the type of Fig. 4 are obtained. The first rise of the rate of oxygen production on irradiation is somewhat less steep than in curves of the type of Fig. 3; the slope decreases after about ten seconds, and the transition to the final rate is somewhat slower. (We recall that there is a relation between this type of induction curves and the sigmoid-shaped saturation curves and low yields of light flashes.) Our experiments indicate that these induction phenomena occur more often in hydrogen than in nitrogen atmospheres and also that they are more frequent under carbon dioxide limitations. However, we were never able to predict with certainty which type of curve would result. The age of the culture and slight variations in the culture conditions are known to have a decisive influence on induction phenomena. That is understandable since they are sensitive to changes in the concentration of metabolic products and therefore behave erratically unless extreme care is taken to standardize the culture conditions. This was beyond the scope of the present work.

Figure 2 shows the type of curve which most frequently results with *Chlorella*. With *Scenedesmus* we found it at room temperature only once (using an old culture) and several times at low temperature. The initial part of the curve is similar to the beginning of the time curves described above, but after about one-half minute of irradiation the rise of the rate of oxygen production is replaced by a decline; after passing through a minimum the rate again increases until the final steady state is reached. To distinguish the loss of oxygen production during this period from the one occurring at the very beginning of illumination, we will call it the secondary induction loss. Both the extent of the secondary induction loss and its duration depend upon the length of the previous dark period. The curve of Fig. 2 was measured after three hours of darkness. The first measurement represented in Fig. 10 was made after a new dark period of fifteen minutes. The second curve was measured after another dark period of forty minutes. Lowering of the temperature makes the minimum more pronounced and broader (see Fig. 11). Poisoning by cyanide or extreme carbon dioxide limitation also enhance the depth and breadth of the minimum.

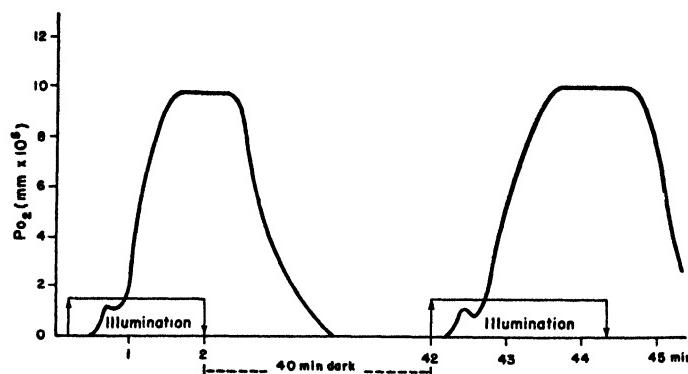


FIG. 10

Time Curve of Oxygen Production by  $7.5 \times 10^{-4}$  g. *Chlorella* in  $2\frac{1}{2}$  cc. Solution after Three Hours Anaerobicity in Nitrogen after 15-Minute Dark Period and 40-Minute Dark Period, Respectively

That a secondary induction period does sometime occur is not a new observation. It was observed in aerobic measurements of the time course of photosynthetic carbon dioxide uptake by McAlister (8) and by Aufdemgarten (9), and the corresponding maximum in the fluorescence time curves was observed with *Chlorella* under aerobic and anaerobic conditions by McAlister and Myers (10) and by Wassink and Katz (11). Nevertheless, according to the explanation given by Franck, French, and Puck (12), it was not necessarily to be expected that such a secondary induction period would occur also in the time curve of the oxygen production. Since this is now an established fact, the time curve of the fluorescence of *Chlorella*, as measured at room temperature in nitrogen by Wassink and Katz (11), may be reprinted here

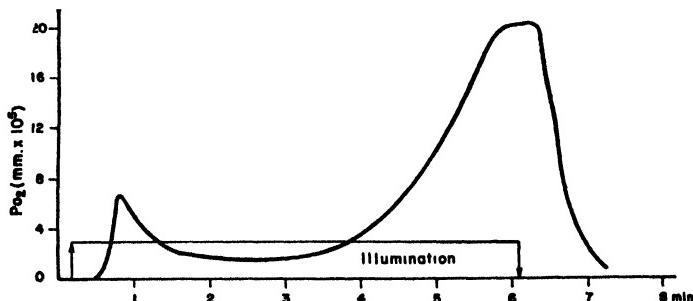


FIG. 11

Time Curve of Oxygen Production by  $17.5 \times 10^{-3}$  g. *Chlorella* in  $2\frac{1}{2}$  cc. Solution at  $0^\circ\text{C}$ .

as a good example of the antiparallelism between photosynthetic activity and fluorescence intensity (Fig. 12).

*B. Influence of Extreme Carbon Dioxide Limitation and of Cyanide on the Time Course of Oxygen Production.* It was mentioned above that, despite the absence of carbon dioxide in the nitrogen stream, a very low partial pressure of carbon dioxide develops by fermentation in the vessel containing the algae. Under these conditions, the rate of oxygen production in light is not constant. During the first three to six minutes of irradiation the rate is several times greater than it is afterwards in the steady state. The secondary induction phenomena may be superimposed on this time course.

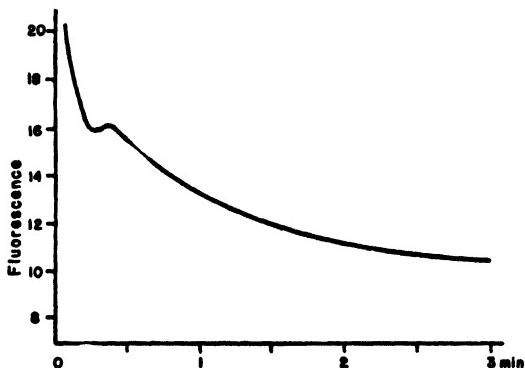


FIG. 12

Fluorescence-Time Relations of *Chlorella* in Nitrogen  
(Reprinted from Wassink and Katz, *Enzymologia*, 1939, 6, 3.)

Figures 13 and 14 are examples of a curve of this type measured with *Scenedesmus* at 0°C. and 12°C., respectively. The general character of curves taken under similar conditions with *Chlorella* is the same, only the secondary induction minimum is more pronounced. The difference between the rates measured during the first minutes and the final rates depends upon the length of the preceding dark period. It is very small after dark periods lasting a few minutes and rises slowly with the duration of the dark pause. After several hours of darkness, we observed that the maximum rates in the first minutes were three to eight times greater than the final rates.

The same change of the time course of oxygen production which

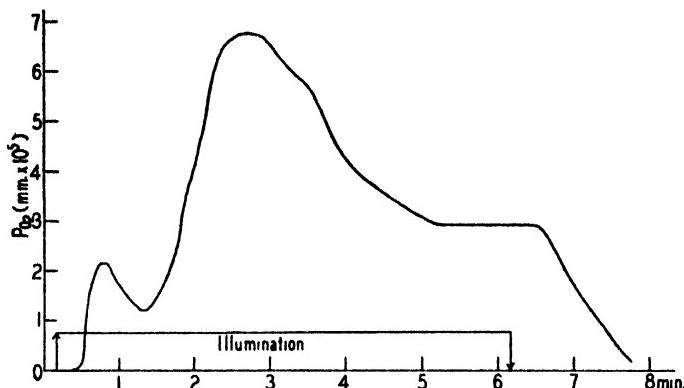


FIG. 13

Time Curve of Oxygen Production by  $12.5 \times 10^{-3}$  g. *Scenedesmus* in  $2\frac{1}{2}$  cc. Solution at  $0^\circ\text{C}$ . with No Additional Carbon Dioxide

occurs under severe carbon dioxide limitation also occurs if the carbon dioxide concentration is abundant but if its uptake is limited by the poisoning of the carboxylating catalyst (catalyst A in the theory of Franck and Herzfeld) by cyanide. In this case, too, the rate of oxygen production is considerably greater in the first minutes of irradiation

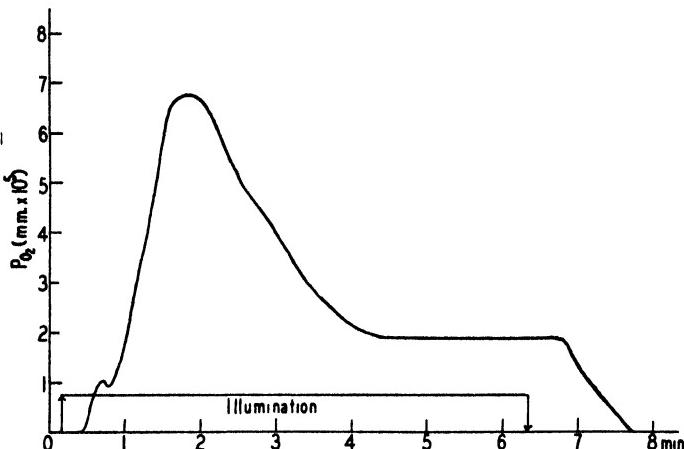


FIG. 14

Time Curve of Oxygen Production by  $12.5 \times 10^{-3}$  g. *Scenedesmus* in  $2\frac{1}{2}$  cc. Solution at  $12^\circ\text{C}$ . with No Additional Carbon Dioxide

than in the steady state. This difference between the initial and final rates is even greater than that caused by carbon dioxide limitation. There is practically no difference to be observed in the first part of the time curves, whether cyanide is present or not, while the final rate is appreciably reduced by cyanide.

Examples of the result of measurements with *Scenedesmus* at 0° in the presence of  $10^{-3} M$  HCN are given in Fig. 15. These curves also

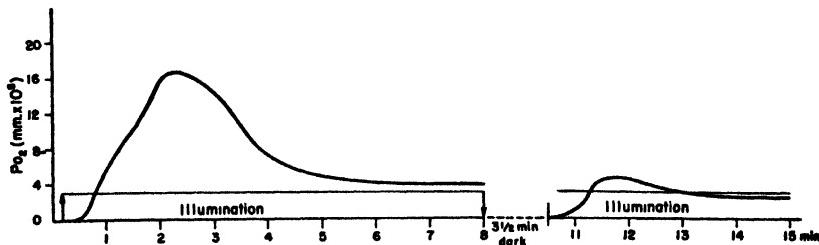


FIG. 15

Time Curve of Oxygen Production by  $12.5 \times 10^{-3}$  g. *Scenedesmus* in  $2\frac{1}{2}$  cc Solution at 0°C in Presence of  $10^{-3} M$  HCN

demonstrate the influence of the preceding dark time. In these measurements the partial pressure of carbon dioxide was twenty mm.

### III. Oxygen Production by Single Light Flashes

Up to the present time, all the work on the yield of photosynthesis by light flashes has been done by observing the integrated effect of a long series of flashes separated from each other by dark pauses lasting between 0.004 and 0.2 sec. This method proved to be a very powerful tool for studies of the interaction of photochemical steps and catalytic dark reactions in photosynthesis, but some problems concerning the yield per flash are still open and can hardly be solved by that method. Thousands of flashes are necessary to produce an effect measurable with the methods hitherto employed, and it is difficult to make the intensity of the individual flashes strong enough to reach flash saturation. In Emerson and Arnold's (3, 13) experiment it was just possible to reach saturation intensity in the presence of a surplus of carbon dioxide. According to Franck and Herzfeld the maximum yield per flash should be independent of carbon dioxide concentration, but the flash saturation intensity should lie higher if carbon dioxide is limiting.

If that conclusion is right, Emerson and Arnold's observation of the lowering of the maximum yield per flash by carbon dioxide limitation should indicate only that the necessary saturation intensity was not reached under these conditions. The present method, which is sensitive enough to measure the oxygen produced by a single light flash, should be able to settle that question, since there is no difficulty in raising the light output of single flashes. (The total light output of the flash lamps used is 25,000 *lumen* seconds, and the peak intensity, 1,400,000 *lumens*, cf. Fig. 6.)

Another question of considerable interest is whether the yield per flash calculated from the effect of the long succession of flashes has the same order of magnitude as the yield produced by a single individual flash. The reasons why that is by no means self-evident will be given in the discussion.

Using the present method, the solution to the first mentioned problem is possible, providing all other conditions influencing the yield can be kept constant while the carbon dioxide concentration is changed; the answer to the second is not so simple. As we have seen, photosynthesis by algae under anaerobic conditions is inhibited by a poison whose concentration depends on the concentration of algae. To compare the flash yield from our experiments with those from the literature, we must ascertain, therefore, that the flash yield is practically uninfluenced by this poison and, furthermore, that it is reasonably free from influences causing induction losses.

Accordingly, the oxygen yield per flash was measured with *Chlorella* and *Scenedesmus* under a variety of conditions. As mentioned in Chapter I, the flash illumination was produced by igniting photoflash bulbs in the immediate neighborhood of the algae. It may be seen from Curve 6 that the light intensity produced is many times greater than that of the brightest flashes used by Emerson and Arnold. Thus, even under severe carbon dioxide limitations flash saturation was certain. The duration of the flash is about twice as long as the working period of the catalyst B (which, according to the theory of Franck and Herzfeld, is measured by Emerson and Arnold's method); at 0° this method measures another catalytic period, which is much longer than the flash duration, but the working period of catalyst B is also prolonged so as to be much longer than the duration of the flash (see *Discussion*). Therefore, for comparing absolute yields, only those measurements made at low temperature are reliable; where only

relative values are required, *e.g.*, in the search for an effect of concentration, both the experiments at low and at high temperatures can be used.

All measurements have been made after three hours of anaerobic treatment. Thus, time is sufficient to render the cells free of oxygen; longer anaerobic periods were avoided because they might cause stronger photosynthetic limitations by the development of both catalytic and narcotic poisons.

The main results are the following: With concentrations of  $5 \times 10^{-3}$  g. of algae per cc. or higher, the burst of oxygen caused by a single light flash is easy to measure. The total output is determined by graphical integration of the time course of oxygen production by the flash. With concentrations equal to or lower than  $5 \times 10^{-4}$  g. per cc., the integration becomes inaccurate since the burst is small. Within the limits of the errors of observation, the oxygen production seems to be proportional to the number of algae. In other words, no dependence of the yield upon concentration was found, unlike the behavior of the saturation rate observed with continuous irradiation. This fact does not contradict the conclusion that the main limitations of the saturation are imposed by the inhibition of some enzymatic process. It shows only that the enzyme which is poisoned is not the one responsible for the occurrence of flash saturation, *i.e.*, not the catalyst B, in the theory of Franck and Herzfeld.

If several flashes are used successively—separated by dark pauses of about one min.—both primary and secondary induction losses are easily detectable. The curves of Fig. 5a, b are examples of the oxygen production by such a series of flashes in suspensions of *Chlorella* or *Scenedesmus*, respectively. In both strains of algae, the first light flash usually produces a lower yield than the second or the third, indicating a primary induction loss. In suspensions of *Scenedesmus* after a few flashes the yield remains constant; in suspensions of *Chlorella* the yield falls again, closely following the pattern of the secondary induction loss shown by time curves in continuous light. (For practical reasons, only the declining part of the secondary induction phenomenon was measured—an extension beyond the minimum would have consumed too many photobulbs.) The primary induction loss varies in its extent; for instance, it is not perceptible in the example chosen in Fig. 5a. Occasionally, the primary induction loss becomes so large and so persistent that the yield per flash remains

very small, even after a long series of flashes and brief periods of continuous illumination. As was mentioned in the two preceding chapters, the occurrence of such low flash yields is associated with the sigmoid shape of the saturation curve and a slow approach to the steady state of the time curves. Even if one excludes these comparatively rare cases, the yield per flash is somewhat variable. For quantitative comparisons only the final values observed with *Scenedesmus* suspensions have been used and, as a further precaution, one and the same suspension was used in a quick succession of measurements whenever the influence of the variation of an external factor was studied. In this way it was found that the yield per flash is somewhat greater at room temperature than at 0°C. and is also higher at room temperature if carbon dioxide is abundant as compared with the yield at the same temperature in absence of an external source of carbon dioxide. Both results are a consequence of the fact that at room temperature the duration of the light flash exceeded the working time of the critical rate limiting catalyst, whereas at 0°C. it was smaller than the latter. Experiments carried out at 0°C. showed that (as predicted by Franck and Herzfeld (14)) the yield per flash is independent of the carbon dioxide concentration. The average oxygen yield obtained from a flash illumination of a suspension of *Scenedesmus* was  $2.3 \times 10^{-8}$  cc. if no carbon dioxide was mixed with the carrier gas and  $3 \times 10^{-8}$  cc. if additional carbon dioxide was present. The difference lies well within the possible experimental error.

Measurements at 0°C. have also been used to compare the yield per flash measured by the present method with the yield measured aerobically. The order of magnitude of the yields turned out to be the same, but the values measured have regularly been five to ten times smaller than in air. Emerson and Arnold compared the number of oxygen molecules evolved per flash with the number of chlorophyll molecules present, and found that in *Chlorella* the ratio

$$\frac{\text{number of oxygen molecules}}{\text{number of chlorophyll molecules}} \approx \frac{1}{2000}.$$

Our best anaerobic yields with *Scenedesmus* corresponded to a ratio of about  $\frac{1}{10,000}$ . (Measurements with *Chlorella* have not been used for the comparison because of the secondary induction losses.) If one takes into account that small losses of oxygen are possible, since

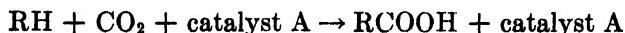
even under optimum conditions some narcotic poison is formed under anaerobic conditions, the difference between the two figures is insignificant; it is actually not greater than those found between different species under aerobic conditions (Arnold). (13.)

## PART Two—DISCUSSION

As main basis for the interpretation of the results the following papers have been used: Franck and Herzfeld's theory of photosynthesis (14), Gaffron's papers on photoreduction (4), and Franck, French, and Puck's interpretation of the induction period (12). The reason for using the latter two as fundamental investigations lies in the fact that they are the only ones available in the literature for that purpose; furthermore, the phenomena presented here are in good agreement with their theoretical content. Franck and Herzfeld's theory is chosen as the one which is in no contradiction with the experimental results published in the literature, and fits the new results of this paper as well. The only other theory which has been worked out in considerable detail and in different forms, the theory of the photosynthetic unit (15), contradicts several old observations as well as some contained in this paper. These remarks do not mean that the authors expect each assumption of Franck and Herzfeld's theory to prove correct in the future (in particular, the chemical equations of that theory were meant and characterized as model equations to be replaced in due time when more pertinent information will be available) but they believe that the general structure of that theory is sound and that the new observations give it additional strength.

Throughout the discussion we will make free use of the nomenclature defined in Franck and Herzfeld's paper. While it is impossible to repeat its content here (the reader may be referred to the original literature and the surveys written on this subject, 4, 16), it may be of help to indicate very briefly the functions of the enzymes, which, according to that theory, are responsible for the light saturation rates of photosynthesis under various conditions:

1. Catalyst A is the symbol for the enzyme involved in the reaction:



in which RH is a colorless organic molecule of molecular weight about 1000 (18), and RCOOH is the CO<sub>2</sub> complex which is reduced photo-

chemically. This reaction becomes rate limiting for photosynthesis (a) in presence of cyanide, which poisons catalyst A; (b) at low temperatures, since the reaction has a high temperature coefficient; (c) if the partial pressure of  $\text{CO}_2$  is too low.

2. Catalyst B is the symbol for an enzyme involved in the transfer of hydrogen atoms from the hydrogen donor to the  $\text{CO}_2$  complex. It is furthermore assumed that the same enzyme is used for the restoration of the dehydrogenated hydrogen donors by a transfer of a hydrogen atom from the water. The energy needed for the two sets of reactions, each comprising four steps, is provided by light quanta absorbed by the chlorophyll. It is therefore necessary that catalyst B be available for work at the right spot immediately following the light absorption act by the individual chlorophyll molecules. If it arrives too late or if it is still engaged in the working period of a previous reaction cycle, the light energy absorbed is lost by back reactions, and it is transferred into heat movements. These reactions steered by catalyst B are responsible for the maximum rate of photosynthesis, provided that none of the conditions prevail under which catalyst A is limiting. The maximum yield of photosynthetic gas exchange caused by a single light flash is a measure of the number of catalyst B molecules present in the chloroplasts.

3. The symbol, catalyst C, represents the enzyme or the enzyme system which is used for the liberation of oxygen from the photo-peroxides. Under aerobic conditions it is not limiting at any temperatures, but it becomes partly deactivated if the plants (especially higher plants) are kept in the dark for periods of minutes or longer. This deactivation of catalyst C is responsible for the anomalies of the photosynthetic rate during the induction period. Catalyst C also becomes limiting if specific poisons like hydroxylamine, *o*-phenanthroline, etc., are present.

We present first the reasons why we believe that the complications introduced into the process of photosynthesis by anaerobiosis can be explained by two factors: the specific inhibition of the oxygen liberating system and a general narcosis by products of fermentation. Neither of these assumptions is new. That the photosynthetic activity is reduced by anaerobic incubation has been known for a long time. Gaffron (4), who studied this question, concluded that two phenomena are responsible for the smaller photosynthetic rates at light saturation under anaerobic conditions: a general inhibition of the photochemical

processes by products of fermentation and a reduction of the oxygen production by a reaction of the photoperoxides (intermediate oxidation products from which molecular oxygen is liberated in the last step of photosynthesis) with hydrogen-containing substances or with molecular hydrogen. If the photoperoxides are reduced by such a reaction, they are prevented from evolving oxygen with the help of the oxygen-liberating enzyme, catalyst C. In some strains of algae—*Scenedesmus* is one of them—the reduction of the peroxides can be carried out by molecular hydrogen with the help of the enzyme hydrogenase. In this case, "photosynthesis" is replaced by "photoreduction." No oxygen production is observed manometrically during photoreduction, but irradiation with strong light causes the transition to photosynthesis with normal oxygen production.

The present results confirm Gaffron's point of view that two factors are responsible for the inhibition of oxygen production by anaerobiosis and add new facts about the way it is achieved.

### I. Saturation Rates and Saturation Curves

The most striking result obtained by the present method is the reduction of the saturation rate of oxygen production measured anaerobically as compared with the aerobic rate. The factor by which it is reduced becomes as high as 10,000 after long anaerobic incubation if the concentration of algae is high, and can be as low as ten after a few hours anaerobiosis if the algal concentration is exceedingly low. At low temperature and in presence of cyanide the reduction factors are smaller. Furthermore, the factor is about the same whether the carrier gas is nitrogen or hydrogen, and some oxygen production is detectable at all light intensities in both gases.

We discuss first the connection of the present results with photoreduction: Two of the present observations seem to be in contradiction with Gaffron's results. The first is the development of oxygen by *Scenedesmus* in a hydrogen atmosphere, even at low light intensities and with concentrations of algae just as high as those used for manometric measurements; the second is the failure of strong irradiation to effect a transition to a normal rate of oxygen production. A comparison of the principles of the manometric method and of the present one reveals the reasons for the difference of results.

In the manometric measurements any oxygen evolved accumulates in the vessel unless it is consumed again by secondary reactions. If

oxygen is evolved at a very small rate during photoreduction at low intensities, it is not detectable with a manometer, unless it accumulates over very long irradiation periods; and it can accumulate only if it is not consumed. Oxygen is taken up, for example, by the oxyhydrogen reaction, which has been shown by Gaffron (4) to occur in *Scenedesmus* after anaerobic incubation in hydrogen. An accumulation will take place only when the rate of oxygen production surpasses the rate of its consumption. In the present method, on the other hand, no oxygen accumulation can occur, since the gas evolved is quickly carried away by the stream of oxygen-free hydrogen (or nitrogen), and the oxygen which is produced remains in contact with the algae for so short a time that the oxygen uptake by the oxyhydrogen reaction is exceedingly small. That, together with the extreme sensitivity, makes it possible to observe the very small rates of oxygen production not detectable by the manometric method. The same explanation holds for the non-occurrence of the reversion to normal oxygen production by strong irradiation. With the present method, even if the rates of oxygen production rise with the light intensity, the partial pressure of oxygen is maintained at a very low level (less than  $10^{-3}$  mm.). Consequently, the anaerobic conditions remain practically fulfilled even at light saturation. Gaffron has shown that the transition from photoreduction to photosynthesis is a complicated process in which hydrogenase is deactivated and the oxygen liberating system is revived. According to his results, as long as the algae are not illuminated, no deactivation of hydrogenase is caused by oxygen, even if its pressure is as high as 20 mm. Since the method employed is able to detect only oxygen and no other gas, no conclusions can be drawn from the present experiments regarding the action of hydrogenase and the uptake of hydrogen and carbon dioxide. A combination of this method with measurements of the rate of carbon dioxide uptake would be of interest for detailed studies of the transition from photoreduction to photosynthesis.

If one accepts the point of view that the oxygen-liberating enzyme (catalyst C) alone becomes poisoned by anaerobic incubation, none of the experiments appear to contradict this assumption. We present briefly the evidence for that point of view. Gaffron's conclusion that photoreduction differs from photosynthesis only in the different use made of the photoperoxides was strongly supported by Rieke's (17) work which proved that anaerobic photoreduction with hydrogen

proceeds in *Scenedesmus* with the same optimum quantum yield as photosynthesis under aerobic conditions. Consequently, the whole enzymatic system common to both processes must remain unaltered with the exception of catalyst C which is replaced in photoreduction by the hydrogenase. Rieke's experiments show that under the particular conditions of his experiments on quantum yield no unspecific effects (which may briefly be called narcotic influences) were present in spite of the absence of oxygen, since narcotics would have limited the use of the light energy absorbed by chlorophyll for all photochemical purposes, by covering the surface of the chlorophyll-protein complexes and would thus have reduced the maximum quantum yield of photoreduction. The present paper confirms these conclusions, as shown by the following considerations.

The enzyme (catalyst A) which is involved in the uptake of carbon dioxide in the dark is not inactivated by anaerobiosis. That can be deduced from the observation that the factor by which the oxygen production is reduced by anaerobic conditioning is comparatively small at low temperatures and in the presence of cyanide. The arguments are as follows: Franck and Herzfeld have suggested that the temperature coefficient of the carboxylation reaction which is catalyzed by catalyst A is higher than that of all the other catalytic reactions in photosynthesis and that, therefore, this reaction becomes rate-limiting at 0°C. If catalyst A would be the enzyme which is poisoned by deprivation of oxygen, the carboxylation reaction in this case should become limiting even at room temperature. In that case, the anaerobic saturation rate of oxygen production would have the same high temperature coefficient between room temperature and 0°C. as was attributed to the carboxylation reaction. The temperature influence on anaerobic oxygen production would then be rather larger than that of aerobic photosynthesis, while it is, on the contrary, much smaller. We conclude, therefore, that catalyst A is not poisoned by anaerobic treatment. This conclusion presupposes that if a poison inactivates catalyst A, the same percentage of this catalyst should be poisoned at 0° as at room temperature. Analogous conclusions can be drawn from the relative insensitivity of the anaerobic saturation rate of oxygen production to cyanide, since catalyst A is the one which is definitely known to be particularly sensitive to cyanide. A poisoning of the catalyst B (to which the stabilization of intermediates is ascribed in our theory) cannot be responsible for the anaerobic limitation,

since the amount of oxygen produced by a single light flash of saturating intensity (14), which can be considered as a measure of the number of active catalyst B molecules, is of the same order of magnitude under anaerobic conditions as it is in air, while the saturation rate in continuous light is reduced by anaerobiosis by a factor of one thousand or more. Furthermore, no dependence of the flash yield on the concentration of algae could be detected, in contrast to the case of continuous irradiation. (The slight reduction of the yield per flash with increasing concentration of the algae can easily be explained by narcotic influences which will be discussed below.) The total amount of peroxides produced by a single light flash is small enough to be handled by the active part of catalyst C, even if most molecules of this catalyst are inactivated by anaerobiosis.

Since neither catalyst A nor catalyst B is influenced by anaerobic treatment, an inhibition of catalyst C is the only remaining alternative in the frame of the Franck-Herzfeld theory. If no hydrogenase is present (as in the case of *Chlorella*) or if no hydrogen is available the peroxides which are not removed quickly enough by catalyst C will react with organic substances oxidizing them stepwise to carbon dioxide and water. An indirect evidence that the photoperoxides are eliminated is offered by the fact that no accumulation of peroxides is detectable by the phosphorescence method. If the peroxides would accumulate and survive, the oxygen production should continue for some time after the light is shut off. For instance, if only 1/1000 of all the peroxides produced per unit time can be handled by the fraction of catalyst C molecules which are still active even under anaerobic conditions, the oxygen production should last one thousand times longer than the illumination period. Actually, after the light is turned off, the quenching effect on the phosphor falls off as quickly as is to be expected from the inertia of the apparatus. The peroxides can thus survive for not more than a few seconds. An analogy to the oxidation reaction which eliminates the peroxide can be found in Franck, French, and Puck's (12) explanation of the normal induction phenomena. There, also, catalyst C is assumed to be partially inactivated, causing the photoperoxides to attack metabolic substances, giving a narcotizing substance as an intermediate oxidation product. We shall come back to this picture in the discussion of the transient phenomena.

Next, we have to discuss the scanty information concerning the way in which the inhibition of catalyst C by anaerobic incubation

takes place. The substance which poisons catalyst C is unknown. The dependence of the inactivation upon the concentration of the algae indicates that by the anaerobic metabolism a water-soluble product is formed which penetrates the walls of the cells. It is uncertain whether this substance is itself the specific poison which inactivates catalyst C, or whether the substance changes the anaerobic metabolism in such a way that a specific poison is produced metabolically. Experiments on the influence of dilutions of the suspension with fresh water, which have been made but which were not conclusive enough to be presented here, made the impression that the last-mentioned mechanism is the more probable. It is not impossible that the substance is identical with Pratt's (19) chlorellin, an inhibitor of photosynthesis, which, according to Pratt, is produced by old cultures of these algae. Pratt's inhibitor, just as the inhibitor found by us, is water-soluble and its concentration depends upon the concentration of the algae in the solution. The fact that the inhibiting substance found in our experiments is destroyed in air, while chlorellin can be produced aerobically, is no proof against their identity. It is not known whether in our case the poison is destroyed directly by oxygen, or by aerobic respiration. If the latter is the case, the respiration in young and old algae may be different enough to account for the accumulation of the poison in one case and its quick removal in the other.

The discussion so far has been devoted to the explanation of the main factor responsible for the reduction of the *saturation rate* by anaerobic conditions. The same factor influences the *shape of the saturation curves* of the *Type I*. They have all the characteristics of saturation curves taken in presence of an enzymatic poison. The saturation rates are depressed much more than the rates at low light intensities, and saturation occurs at light intensities much lower than in air.<sup>4</sup>

In Section II, examples were given showing how, if the duration of

<sup>4</sup> While that is, in general, typical of the poisons which influence enzymes, it is not true if catalyst C is poisoned under aerobic conditions. For instance, hydroxylamine, which is known to be a poison for catalyst C, reduces photosynthesis in air about equally at all light intensities and, according to Franck and Weller, the flash saturation rate in air is reduced by about the same amount. According to Franck, French, and Puck (12), and to Franck and Weller (13) this exception is a peculiarity of the aerobic interplay between activation and deactivation of catalyst C but does not apply to the anaerobic conditions under which a poisoning of catalyst C should have the usual influence on the shape of the saturation curve.

anaerobiosis is prolonged, the saturation curve undergoes a typical change which is coupled with a further reduction of the saturation rate. Occasionally, and more often in hydrogen than in nitrogen, the sigmoid-type of saturation curve is fully developed after only three to four hours of anaerobic treatment. Obviously, an inhibiting effect of a different kind, superimposed on the one already discussed, is revealed by these changes. This second inhibitory effect especially depresses the rates at low light intensities and shifts the intensity needed to reach saturation to higher values, approaching those required for saturation under aerobic conditions. A clear indication of the nature of this inhibition can be found in the observation that the occurrence of sigmoid saturation curves goes hand-in-hand with the development of a prolonged induction period and with a depression of the yield of oxygen produced by single light flashes. All these phenomena indicate the presence of an inhibitor of the type of a narcotic. Farther below, when the transient phenomena and their connection with the time course of fluorescence will be considered, evidence will be given that during the anaerobic dark metabolism there is often produced a narcotizing substance which covers a large part of the chlorophyll, thereby inactivating it, and apparently also inhibits to the same extent the function of the catalyst B (probably by being adsorbed on its protein). No observations are known which decide whether catalyst A and catalyst C are also affected by this *narcoticum*. The narcotic substance can be removed by oxidation. Under aerobic conditions, this removal is brought about by respiration (15); under anaerobic conditions by a reaction with the photoperoxides. Therefore, if present in our algae, the *narcoticum* must have the greatest concentration at the low light intensities and must disappear with rising light intensity until saturation is reached. As a result, photosynthesis rises quicker than linearly to its saturation rate. It may be noted that the sigmoid saturation curve is usually observed for photoreduction in purple bacteria (20) as well as in *Scenedesmus* (17). Of special interest is the coupling of the decline of the saturation rate with a rise of the saturating light intensity until the latter approaches the saturation intensity observed under aerobic conditions. For the interpretation of this relationship an analysis of the conditions is necessary. In the absence of a *narcoticum*, saturation of the oxygen production is reached when as much peroxide is produced as the strongly inhibited catalyst C can handle; but the peroxide production

itself continues to rise with the light intensity until its own saturation is reached. The latter lies at the much higher light intensity, identical with the one needed for saturation under aerobic conditions. (The maximum peroxide production which is determined by the efficiency of the catalyst B is the rate-limiting reaction under aerobic conditions.) The saturation intensity for the peroxide production is not influenced by the presence of the *narcoticum*, just as the saturation intensity of aerobic photosynthesis remains unaltered if the saturation rates are reduced by a *narcoticum*. If, now, catalyst C is less influenced by the *narcoticum* than is catalyst B and the chlorophyll, the oxygen production will rise with the rising peroxide concentration either until all *narcoticum* is removed by the reaction with the peroxide or, if not all *narcoticum* can be removed, to the saturation point of the peroxide production. In most observations the first is true since the saturation intensity for the oxygen production remains below the limit indicated by aerobic photosynthesis. The reduction of the saturation rate of the oxygen production, induced by prolonged anaerobiosis, must be attributed in these cases entirely to a rise of the level of the specific poison for catalyst C. On the other hand, in the experiments carried out with the very high concentration of  $9 \times 10^{-3}$  mg. of algae per cc. in an atmosphere of hydrogen (Fig. 9) the saturating light intensity is identical with that in air (within the limits of the errors of observation) even after as little as four hours of anaerobiosis, and no further shift to higher values occurs if the anaerobic period is prolonged. The conclusion is, therefore, that the limitations of the saturation rate of oxygen production under the conditions of these last experiments are imposed by both the catalyst C poison and the *narcoticum*. There are, indeed, indications that the level of the *narcoticum* can be high at saturation intensities after prolonged anaerobic treatment, if the algae concentration is high: The observations of Willstätter (21), Gaffron (22), and Noack, *et al.* (6) on extreme limitations of photosynthesis in higher plants and in algae after twenty hours of anaerobic incubation can be quoted as such indications. Sometimes, photosynthesis does not start at all in spite of strong irradiation, while in other cases irradiation from ten minutes to one hour is required to restore the photosynthetic activity. Noack and co-workers, using the manometric method observed only a brief induction period with *Chlorella* after twenty hours of anaerobiosis, if the suspension was alkaline, but found photosynthesis to be

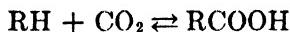
suppressed entirely in acid solution. In our experiment, changing of the pH did not produce systematic differences in excess of natural fluctuations of the results. On the other hand, very small changes of the concentration of the *narcoticum* can be responsible for starting or non-starting of photosynthesis, if the latter is measured in a closed vessel. If the oxygen production in Noack's manometric vessel is smaller or equal to the oxygen consumption, no photosynthetic activity observable with the manometer will result, but if the production surpasses the consumption even by the smallest margin, the partial pressure of oxygen will start to rise, and will revive the photosynthetic activity autocatalytically (cf. p. 130).

## II. *Transient Phenomena*

A. *Induction Phenomena*.—An extensive discussion of the induction phenomena is not necessary, since most of the phenomena observed with the present method were predictable from the earlier measurements of the development of fluorescence under anaerobic conditions by using the well established rule of antiparallelism of fluorescence and photosynthesis. The principal features of the induction phenomena and of their interpretation (according to Franck, French, and Puck (12)) under aerobic conditions are the following: In air the *narcoticum* is absent at the beginning of illumination, but metabolic products are present from which *narcoticum* can be produced by a reaction with the photoperoxides. This is what happens at the beginning of the illumination period, because catalyst C is partially inactivated during the preceding dark period and is, therefore, unable to remove the peroxides quickly enough at the start of the illumination to prevent the reaction with the "precursor" of the *narcoticum*. For algae in air, the concentration of the precursor is low and catalyst C is less strongly inactivated than in higher plants. The induction phenomena are, therefore, less pronounced than in leaves (9, 10). As the products of photosynthesis are accumulated, catalyst C becomes reactivated and the *narcoticum* is removed through respiration. In addition to the primary induction losses, there often develops, e.g., in *Chlorella*, a secondary induction minimum, which is not yet satisfactorily explained. The fluorescence time curves under anaerobic conditions have been extensively studied only in the case of the algae *Chlorella* (11) (23) and *Scenedesmus* (Shiau, 23). The main difference between these curves (as compared with the ones measured under aerobic

conditions) consists in the fact that the fluorescence intensity is high at the very start of the illumination period. With *Scenedesmus* only a very small additional rise of the intensity occurs during the first few tenths of a second followed by a decay, while with *Chlorella* the first rise is more pronounced but still much smaller than under aerobic conditions. The conclusion is obvious that the major part of the *narcoticum* is present at the very beginning of the illumination and that this part is a product of the anaerobic dark metabolism. The amount of the narcotic made metabolically is variable. In some cases (of which Curve 12 is an example) the concentration of the narcotic is high, as indicated by the fact that the fluorescence intensity starts much higher in  $N_2$  than in air and decays slowly to a final value, which is identical with the one observed in air. In other cases, the concentration of the narcotic is apparently small, as indicated by small differences in intensities in aerobic and anaerobic fluorescence time curves. The decay of the fluorescence intensity shows that the narcotic is partially or totally removed at the end of the induction period; if oxygen is allowed to accumulate, the removal is caused by respiration as in air, but if the oxygen partial pressure is kept very low by a stream of oxygen-free gas, the removal has to be accomplished by oxidation of the narcotic through a reaction with the photoperoxides. A secondary fluorescence maximum, which corresponds to the secondary induction loss in the rate of oxygen production, is often superimposed on the general decay. According to results of Shiao, the secondary maximum becomes more pronounced if the period of anaerobicity is prolonged. Every one of the fluorescence anomalies has its counterpart in an anomaly in the rate of oxygen production described in the experimental part of this paper. It may be mentioned that the correspondence of the secondary maximum in the fluorescence to a minimum in the oxygen production was not foreseen in the paper of Franck, French, and Puck. These authors thought it possible that the peroxides themselves might accumulate if they are not quickly removed by catalyst C, and that the peroxides might displace intermediates of photosynthesis from the chlorophyll, enhance the fluorescence, and in that way reduce the  $CO_2$  uptake. This assumption is now ruled out because an accumulation of peroxides cannot be responsible for a minimum in the oxygen production. The problem of the secondary induction phenomenon will be further discussed on a later occasion in the dissertation of Shiao.

*B. Time Curves of Photosynthesis under Carbon Dioxide Limitation and in the Presence of Cyanide.* Of greater general interest are the time curves obtained under extreme carbon dioxide limitation and after the addition of cyanide in the presence of excess carbon dioxide. We shall discuss the latter first. If cyanide is added in a concentration high enough to strongly suppress the photosynthetic rate, the oxygen production is just as high for a couple of minutes as if the poison were absent (cf. Fig. 15); then it declines to a steady rate which is much lower. Cyanide is known to poison the uptake of carbon dioxide by inactivating the catalyst A involved in the carboxylation (14, 18). But as long as not all molecules of catalyst A are deactivated, the establishment of the equilibrium of the reaction



in the dark should remain possible, even though we know experimentally the time required to reach this equilibrium becomes longer if cyanide is added.

Presence of cyanide, therefore, should not change the amount of carbon dioxide complexes at the beginning of a new irradiation period. Consequently, the inhibition by cyanide becomes observable only after the carbon dioxide complexes and intermediates present at the beginning of the irradiation period have been consumed. The retardation of the new uptake of carbon dioxide as compared with the rate of its photochemical consumption is then responsible for the low steady rate of oxygen production.

The fact that the rate of oxygen production at the beginning of the irradiation after long dark periods is as high in the presence of cyanide as it is in its absence, is, therefore, in complete accordance with expectation. The same is true of the duration of the burst, which is in accordance with the value estimated from the concentration of carbon dioxide complexes and intermediates of photosynthesis expected to be present. The amount of the carbon dioxide acceptor in the cell as estimated by Ruben, Kamen, and co-workers (18) is of the same order of magnitude as the amount of chlorophyll present and the amount of partially reduced intermediates should be several times greater. Both together form a reservoir of substances to be transformed by light sufficient to feed the photosynthetic process for a couple of minutes if it proceeds with the normal aerobic saturation rate. In the present case, the integrated surplus of oxygen developed during

the burst is, of course, several hundred times smaller than the number of chlorophyll molecules present, because only a fraction of a per cent of all the peroxides produced are used for the oxygen production because of the anaerobic inactivation of catalyst C.

It is remarkable that dark periods lasting one hour or more are necessary to attain the maximum burst of oxygen at the beginning of the irradiation period. That is in agreement with the results obtained by Kamen, Ruben, and co-workers (18), who found that the formation of RCOOH was greatly slowed down by cyanide but is, on the other hand, in apparent contradiction to McAlister's (8), and especially Aufdemgarten's (9), result that the phenomenon of pickup of carbon dioxide in the dark in presence of cyanide has a duration of only  $\sim$  one minute. The process of the carbon dioxide uptake is a complicated one and by no means entirely understood. If Ruben's (24) point of view is accepted that the carboxylation reaction is tied up with the metabolism (by phosphorylation) it may be inferred that the amount of the acceptor available for carboxylation increases slowly during the dark periods. On the other hand, it is just as possible that a fermentation product whose concentration rises slowly during the dark period, can be used for photosynthesis.

Revealing, in that respect, are the experiments with nitrogen under extreme carbon dioxide limitations. Under these conditions too, a burst of oxygen occurs at the beginning of the irradiation period; magnitude of this burst rises for hours with the duration of the preceding dark period. We may expect that the dark equilibrium concentration of RCOOH should be very small under these conditions. If that point of view turns out to be correct, the surplus oxygen produced in a "burst" after long dark periods has to be produced by photosynthesis from a fermentation product.

### III. Oxygen Production by Single Light Flashes

In the experimental section we have compared the oxygen production measured for a single light flash with the yield per flash calculated from the oxygen production by a long series of flashes, and have mentioned that only the low temperature measurements may be used for this comparison. The reason is that only at low temperatures does the working period of catalyst B last long enough to permit each enzyme molecule to work only once during the illumination by a single flashbulb. The duration of the working period of 0° cannot be

calculated from flash experiments using Emerson and Arnold's method, since under these conditions—in flash light as well as in continuous light—another enzymatic reaction (the carbon dioxide uptake involving catalyst A) appears to be the limiting process. (Weller and Franck, Rieke and Gaffron (13).) Nevertheless, it can be estimated from the temperature coefficient of the saturation rate in air at room temperature (where catalyst B is limiting) that the working period of catalyst B is at least five times longer at 0° than at 25°C.

That is sufficient to fulfill the necessary condition that at 0°C. at least the great majority of B molecules work only once during each of our light flashes. Furthermore, we have shown that the flash yield at low temperature is independent of carbon dioxide limitation. It suffices here to add a few remarks about general conclusions which may be drawn from these experiments.

According to the theory of Franck and Herzfeld (14), the maximum amount of oxygen developed by a single light flash is equal to one-fourth of the number of catalyst B molecules present; experimentally, this number was found to be about two thousand times smaller than the number of chlorophyll molecules. It was supposed, in that theory, that catalyst B has a twofold function, namely, to stabilize both the primary photochemical reduction products and the primary photochemical oxidation products. In other words, catalyst B has to function after each of the eight postulated steps. The reasons why it is regarded necessary to assume a dual function of catalyst B are not connected with the interpretation of the flash experiments. The flash experiments described in the literature can be explained equally well by the assumption that catalyst B functions only either in the first or in the second set of photochemical steps or in both. The new method which measures the oxygen production caused by a single flash proves that catalyst B is certainly connected with the second class, but it cannot be used to settle the question whether catalyst B participates also in the first class of reactions. For that purpose the present results would have to be supplemented by observations of the carbon dioxide uptake caused by a single flash.

#### SUMMARY

The method of measuring very small quantities of oxygen ( $10^{-6}$  mm. Hg partial pressure) by the quenching of the phosphorescence of tryptophavine adsorbed on silica gel has been used to determine the rate

of photosynthetic oxygen production of algae under extreme anaerobic conditions. The algae (*Scenedesmus* and *Chlorella*) were kept for several hours in a slow stream of pure nitrogen or hydrogen before illumination. During the light period the oxygen produced was immediately swept out by the gas stream, so that the partial pressure of oxygen over the algae never exceeded  $5 \times 10^{-4}$  mm.

Time curves of photosynthesis in algae measured under these conditions show secondary induction phenomena similar to those previously observed in fluorescence measurements. Light saturation curves measured in nitrogen or in hydrogen show saturation at lower intensities than those measured aerobically, and the saturation rates of oxygen production are greatly reduced. The reduction factor is variable, depending on the concentration of the algal suspension used; the denser the suspension the stronger the inhibition. Addition of cyanide or deprivation of carbon dioxide have little influence on the evolution of oxygen during the first minutes of irradiation but, as expected, greatly diminish the steady rate of oxygen production. The method is so sensitive that the oxygen released from the algae by a single light flash of about 0.01 second duration can be easily measured. The yield measured for single flashes agrees with that obtained from manometric measurements, in which series of thousands of flashes have been used. Contrary to statements in the literature, it was found that the maximum obtainable yield per flash does not depend on the concentration of carbon dioxide.

These results can best be interpreted by assuming that the anaerobic metabolism gives rise to a water-soluble substance which passes through the cell membranes and acts as an inhibitor to the oxygen-liberating enzyme system. To explain all the data we have to assume in addition that another product of fermentation is found in variable concentration which acts less specifically by inhibiting cell metabolism in general, similarly to so-called narcotics. All the results obtained can be explained on the basis of Franck and Herzfeld's theory of photosynthesis and Gaffron's interpretation of photoreduction. (It can easily be shown that several of them contradict the theories based on the concept of a photosynthetic unit.)

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# **Studies on the Interrelation of Fats, Carbohydrates, and B-Vitamins in Rat Nutrition \***

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Received March 7, 1945

## **INTRODUCTION**

It has been demonstrated in this laboratory (1, 2) that weanling rats fed *ad libitum* rations containing lactose and corn oil grew at a rate inferior to that obtained with butterfat. When glucose was the carbohydrate no difference could be found in rats fed either fat in the presence of lactose. Deuel, *et al.* (3, 4, 5) fed weanling rats an unextracted, mineralized skim milk powder ration to which was added one of the following fats: butter, corn, cottonseed, olive, peanut, soybean, or margarine. They found no significant differences in growth rate, body composition, or pregnancy and lactation performance of rats fed rations containing the various fats. Zialcita and Mitchell (7) also concluded that corn oil and butterfat are essentially equal in growth promoting value for the rat. The paired method of feeding was employed. The ration consisted of 60 parts of ether-extracted skim milk powder and 27 parts of the fat to be tested plus 7 parts of casein, 6 parts of a salt mixture, and fat-soluble and synthetic B-vitamins. Although these investigators did not entirely fulfill the conditions defined by us, the data were interpreted to disprove our original observations.

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by funds granted by the National Dairy Council, Chicago, on behalf of the American Dairy Association; also from funds granted by the Evaporated Milk Association, Chicago. We are indebted to Merck and Company, Rahway, New Jersey, for supplies of the synthetic B vitamins and  $\alpha$ -tocopherol; to Winthrop Chemical Company, New York, for crystalline vitamin D<sub>2</sub>; and to Wilson Laboratories, Chicago, for the whole liver powder.

Deuel and Movitt (6) found that in *free choice* experiments rats chose certain diets containing diacetyl or commercial butter flavoring in preference to the unflavored diet. It was concluded that the superiority of butterfat over certain vegetable oils reported by Schantz, Elvehjem, and Hart (8) "is in all probability to be traced to the fact that weanling rats prefer a butter flavor and will eat more of such a diet." In contrast, Boutwell, *et al.* (2) used the *single choice* technique with *ad libitum* feeding and found that the removal of the flavoring agents from butterfat by chromatography or the addition of one such agent, diacetyl, to corn oil had little or no effect on the average daily food intake and average gain in body weight of rats fed certain purified rations. Flavor was also ruled out because rats showed no difference in food consumption or growth when fed rations in which glucose was substituted for lactose.

In explanation of the superior nutritive value of butterfat over corn oil in certain rations, Boutwell, *et al.* (9) suggested that the substitution of corn oil for butterfat resulted in a decreased synthesis of vitamins by the intestinal flora. It is well recognized that changes in the kind of dietary carbohydrate may be reflected in the amount of vitamins synthesized by the intestinal microorganisms (10, 11, 12). There are no data in the literature which show that the kind of dietary fat may have a similar effect. However, Mannering, *et al.* (11, 13) showed that the isocaloric substitution of fat for dextrin, in a ration restricted in riboflavin content but adequate in other respects, decreased the growth of rats by lowering the bacterial synthesis of riboflavin. There are *in vitro* studies which show that different fats may have varying effects on the growth of bacteria. Strong and Carpenter (14) found stearic and oleic acids were stimulatory to *Lactobacillus casei*. Palmitic and linoleic acids had an inhibitory effect. Feeney and Strong (15) obtained an ether soluble fraction from blood which stimulated the growth of the same organism.

The present study was undertaken to clarify that property of fats which is responsible for the observations of Boutwell and coworkers (9) and to interpret, if possible, the disparity in the conclusions of various investigators (1, 2, 6, 7, 8).

#### EXPERIMENTAL

Male albino rats of the Sprague-Dawley strain (20 days of age, 5 g. weight range) were placed in individual raised screen cages and fed the experimental diets for

six weeks. Food and water were given *ad libitum* each day and food consumption records were kept.

The rations contained carbohydrate 48, purified casein<sup>1</sup> 20, salt mixture<sup>2</sup> 4, and fat (butterfat or corn oil) 28. Three levels of B vitamins were fed as shown in Table I.

TABLE I  
*Composition of Rations*

Component	Medium per cent	Vitamin level	
		High per cent	High plus liver per cent
Carbohydrate	48	48	48
Casein	20	20	20
Salts	4	4	4
Fat	28	28	28
		mg./100 g.	mg./100 g.
Liver extract*			1
Thiamine	0.200	0.500	0.600
Riboflavin	0.300	0.500	0.900
Pyridoxin	0.300	0.625	0.900
Pantothenic acid	1.500	5.0	6.000
Choline	100.0	250.0	200.0
Niacin		0.625	1.000
p-Aminobenzoic acid		30.0	30.0
Inositol		100.0	100.0

\* Wilson's whole liver powder, included at the expense of the total solids of the ration not fat.

These levels are arbitrarily designated as medium (or normal), high, and high plus liver. To insure uniformity among the several rations containing a given level of B vitamins, the proper amounts of the vitamins were incorporated with sufficient casein to enable aliquots to be taken from the same lot of casein for each diet to be mixed. Fat-soluble vitamins were added to both fats so that in addition to the amount naturally present, every 100 g. of ration contained 0.014 mg. calciferol, 0.560 mg.  $\beta$ -carotene,<sup>3</sup> 2.24 mg.  $\alpha$ -tocopherol, and 0.210 mg. 2-methyl-1,4-naphthoquinone. The butterfat was prepared by decantation and filtration of melted (60°C.) fresh unsalted sweet cream butter from the University Creamery. The corn oil (Mazola) was obtained on the local wholesale market. Basal rations without the fat were mixed every three weeks. The complete ration including fat was mixed weekly and kept under refrigeration.

<sup>1</sup> Four extractions of four hours each with continuous stirring; two with boiling ethanol and two with diethyl ether.

<sup>2</sup> Hegsted, *et al.* (16).

<sup>3</sup> Ninety per cent  $\beta$ -carotene. Nutritional Research Associates, Inc., South Whitley, Indiana.

The carbohydrates used were as follows:

Galactose, Pfanstiehl *d*-galactose practical

Glucose, cerelose, a pure commercial monohydrate<sup>4</sup>

Starch, commercial corn starch

Dextrin, corn starch autoclaved 3 hours at 15 pounds, dried and ground

Dextri-maltose, Mead, Johnson and Co., salt-free

Sucrose, commercial cane sugar

Fructose, Pfanstiehl, *d*-levulose, C.P.

$\alpha$ -Lactose, Merck, U.S.P. milk sugar<sup>4</sup>

$\beta$ -Lactose, Borden Company

In one instance the 48 parts of carbohydrate were made up from 24 parts of glucose and 24 parts of galactose so as to represent the hydrolysis products of lactose. In a second case, the 48 parts of carbohydrate were made up from 24 parts of glucose, and 24 parts of fructose, the hydrolysis products of sucrose. The carbohydrate and fat combinations as fed are shown in Table II.

TABLE II  
*Growth of Rats Fed Various Combinations of Fat, Carbohydrate, and Water-Soluble Vitamins (6 Weeks)*

Vitamin level	Medium			High			High plus liver					
	Fat	Butter		Corn		Dif. <sup>2</sup>		Butter		Corn		Dif. <sup>2</sup>
		g.	g.	g.	g.	g.	g.	g.	g.	g.	g.	g.
Galactose												
Galactose-Glucose <sup>1</sup>	185 (3)*	179 (3)*	6	158 (3)*	162 (3)*	-4						
Glucose	172 (12)	175 (12)	-3	180 (18)	182 (18)	-2						
Starch	192 (12)	169 (12)	23	188 (18)	185 (18)	4	212 (12)	209 (12)	3			
Dextrin	187 (6)	169 (6)	18	174 (18)	168 (18)	6	215 (6)	218 (6)	-3			
Dextri-maltose	199 (6)	176 (6)	23	181 (18)	168 (18)	13	205 (6)	202 (6)	1			
Fructose-Glucose <sup>1</sup>	190 (6)	164 (6)	26				200 (3)*	192 (3)*	-3			
Sucrose	185 (18)	148 (18)	37	190 (18)	166 (18)	24	209 (6)	203 (6)	6			
$\alpha$ -Lactose	118 (12)	83 (12)	35	158 (60)	123 (60)	35	167 (12)	145 (12)	22			
$\beta$ -Lactose				141 (18)	118 (18)	23						

\* Except for these groups, all groups consisted of six animals each. The figures in parentheses represent the total number of animals used in computing the average. Figures of 12, 18, or 60 animals indicate the experiment was run twice, three times, or ten times, respectively.

<sup>1</sup> Each of the component carbohydrates constituted 24 parts of the ration.

<sup>2</sup> Positive values indicate the gain made by the butterfat group was greater; negative values indicate the converse.

Growth of weanling rats fed rations containing lactose or sucrose was compared when supplemented with raw pork liver, a very rich source of the B vitamins. No comparison between butterfat and corn oil was attempted with this supplement because, in addition to the vitamins, it supplies appreciable quantities of animal fat.

<sup>4</sup> The water of crystallization was not considered in formulating rations.

The ration had the following composition: lactose or sucrose 46, crude casein 25, salts 4, and butterfat (with added fat soluble vitamins) 25. The high level of B vitamins (Table II) was incorporated in the ration. A supplement of 2.5 g. of fresh, raw pork liver was given daily to each rat. Six weanling male rats were fed each ration *ad libitum* for 6 weeks. The growth and food consumption are shown in Table IV. Over the entire experimental period of 42 days, the fresh liver supplement contributed 31.5 g. of solids to the ration consumed by each rat.

An *in vitro* experiment was made to determine the effect of the free fatty acids of butterfat and corn oil on the growth of *Streptococcus lactis*<sup>5</sup> and *Lactobacillus casei*.<sup>6</sup>

TABLE III  
*Growth of L. casei and S. lactis in the Presence of the Free Fatty Acids of Butterfat and Corn Oil*

Organism	Control tubes	Galvanometer reading* as a measure of growth			
		<i>L. casei</i>		<i>S. lactis</i>	
Fatty acids		Butter	Corn	Butter	Corn
mg./tube		53	85	85	85
0.1		56	103	91	80
0.2		55	108	93	80
0.5		58	101	83	78
1.0		71	96	84	79
2.0		86	92	86	87
4.0		110	99	99	98

\* Corrected for the turbidity of the emulsion of fatty acids. The blank tube was set at 100.

The organisms were grown on the media described by Luckey, Briggs, and Elvehjem (17) made complete by the addition of 0.1 mg. of solubilized liver per tube. The techniques of microbiological assays were followed. The free fatty acids were prepared by accepted procedures with care exercised to prevent oxidation. The lower fatty acids of butterfat were removed by steam distillation. Using a laboratory homogenizer, an emulsion of the fatty acids of butterfat and of corn oil in water was prepared, of such concentration that aliquots could be taken to test varying levels of fat per tube. After inoculation, the tubes were incubated at 37°C. for 16 hours and read turbidimetrically in the Evelyn colorimeter. A series of blank tubes containing the same amount of the fat emulsion and at the same dilution as the experimental tubes was read to determine the correction for the turbidity of the fat emulsion. The data are presented in Table III, growth being expressed in terms of galvanometer reading. The instrument was adjusted so that a blank tube which contained no solubilized liver or fat gave a reading of 100. Growth of *L. casei* and *S. lactis* on the complete media without fat was represented by a reading of 53 and 85, respectively.

<sup>5</sup> Now designated as *S. faecalis* R.

<sup>6</sup> We wish to thank Mr. T. D. Luckey for help in performing this experiment.

TABLE IV  
*Growth and Food Consumption of Rats Supplemented with High Levels  
of B Vitamins Plus Raw Pork Liver*  
(Each figure is the average of 6 male rats over a 6 weeks period.)

Ration	Growth g.	Ration	Total consumption (gm.) Liver supplement*	Total daily consumption
Lactose	215	443	31	11.3
Sucrose	228	450	31	11.5

\* Dry basis.

## RESULTS

An overall summary of the growth data is presented in Table II. All fat-carbohydrate combinations shown within each vitamin level were fed concurrently at least once. However, rations containing different levels of vitamins were fed at different times. Thus the better growth which resulted in certain groups fed the medium level of vitamins as compared to the high level may be attributed to variations in rats from time to time.

In the series fed high vitamin rations plus liver, similar rates of growth were found regardless of the kind of carbohydrate or fat in the ration with the exception of the animals fed lactose. With lactose as the carbohydrate, the group fed butterfat was about 40 g. below the average groups fed the same fat and the other carbohydrates tested. The substitution of corn oil for butterfat caused a further drop in growth of about 20 g. in 6 weeks.

The growth of rats fed the purified rations containing a high vitamin level with no crude vitamin carrier was more variable. Animals receiving galactose as 48 parts of the diet grew rather poorly. This was attributed to metabolic difficulty in utilizing such a level of galactose. All six rats developed bilateral cataract by the fifth week and present investigation (unpublished) indicates that rats fed such a ration excrete a large proportion of the dietary galactose in the urine. There was no difference in the growth promoting value of the two fats on this ration. With the carbohydrate portion of the diet composed of equal amounts of galactose and glucose, good growth was obtained with either fat. No cataract was observed. The groups fed butterfat and glucose-galactose mixture, glucose, starch, dextri-maltose, sucrose, and probably dextrin all maintained superior growth. This was true

also of groups fed corn oil and galactose-glucose mixture, glucose, and starch. A small but definite drop was noted if corn oil was fed with dextrin, dextri-maltose, or sucrose. Again, as was found with high vitamin-liver rations, the growth of animals fed lactose was inferior. In 10 experiments with the  $\alpha$ -lactose, high vitamin ration totaling 120 animals (60 on each fat) the growth on the corn oil diet averaged 35 g. below that obtained on the butterfat regime at the end of 6 weeks. A statistical analysis of the growth data of rats fed rations at the high level of vitamins (Table V) showed the superiority of

TABLE V

*Food Consumption, Growth, and Statistical Evaluation of Growth Difference of Rats Fed Various Fat and Carbohydrate Combinations in Rations Supplemented with a High Level of B-Vitamins<sup>1</sup>*

Carbohydrate	Food consumption		Growth		Growth difference g.	Analysis of variance <sup>3</sup> <i>F</i>
	Butter-fat g./day	Corn oil g./day	Butter-fat g./6 weeks	Corn oil g./6 weeks		
Galactose	9.5	10.1	158	162		
Galactose-Glucose <sup>2</sup>	11.1	10.9	179.8	182.1	2.3	0.06
Glucose	11.2	11.0	187.0	182.8	4.2	0.21
Starch	10.5	10.4	187.6	185.4	2.2	0.06
Dextrin	10.2	10.0	173.9	167.9	6.0	0.43
Dextri-maltose	11.2	10.2	181.1	168.4	12.7	1.95
Sucrose	11.2	10.1	189.7	166.1	23.6	6.72
$\alpha$ -Lactose	10.1	7.7	149.3	123.9	25.4	7.77
$\beta$ -Lactose	10.1	8.2	140.7	118.3	22.4	6.01

<sup>1</sup> These data are taken from the same experiments summarized in Table II, high vitamin column. Each figure is the average of 18 animals, except with galactose as the sole carbohydrate where 3 animals were fed each fat.

<sup>2</sup> Each of the two component carbohydrates constituted 24 parts of the total ration.

<sup>3</sup> The 1% value for *F* is given as 6.72 and the 5% value as 3.87 (one degree of freedom/280 degrees of freedom) in the "Statistical Tables for Biological, Agricultural, and Medical Research" by R. A. Fisher and F. Yates. (Oliver and Boyd, 1938), pp. 31 and 33. We wish to thank Dr. James H. Shaw for help in the statistical analysis.

butterfat was highly significant when either sucrose or  $\alpha$ -lactose was the carbohydrate. These data were well suited for statistical treatment because of the uniform, controlled conditions of the study. In three separate experiments, 96 weanling rats born on the same day and in a 5 g. weight range were divided into groups of 6 and fed 16 different diets. Thus a total of 18 rats represented each diet in the analysis of variance.

Rats fed a medium level of the B-complex and butterfat grew uniformly well on the galactose-glucose mixture, starch, dextrin, the fructose-glucose mixture, and sucrose diets. The corresponding animals on the glucose diet averaged 15 to 25 g. less. With the exception of the galactose-glucose and glucose groups, animals fed corn oil averaged 18 to 37 g. less than the butterfat controls. At this vitamin level, rats grew only 2.8 and 2.0 g. per day when fed the lactose-butterfat and lactose-corn oil ration, respectively.

Rats responded similarly to changes in fat and vitamin level when fed rations which contained either sucrose or the mixture of fructose and glucose equivalent to sucrose on a molecular basis. Lactose, which is considered to be slowly resolved to its component monosaccharides in the intestinal tract, differed greatly from the mixture

TABLE VI

*Food Consumption of Rats Fed Butterfat or Corn Oil in a Lactose High Vitamin Diet*

Days (inclusive)	(Each figure is the average of 6 rats.)	
	Butterfat ration	Corn oil ration
g.	g.	g.
1- 5	21	20
6-10	32	25
15-42	360	265

of galactose and glucose as changes were made in the dietary fat and vitamin level. The greater solubility and apparent sweetness of  $\beta$ -lactose over ordinary milk sugar was not reflected in the growth response of rats. Rather, in three separate experiments  $\beta$ -lactose tended to allow a smaller gain in body weight than  $\alpha$ -lactose. Ershoff and Deuel (21) found  $\beta$ -lactose was more "lethal" to young rats than  $\alpha$ -lactose.

Food consumption was followed in all experiments. The average gain in six weeks and the average daily food intake of rats from one experiment at the high level of B-vitamins are given in Table V. The data show that food consumption parallels growth in all experiments. Typical food consumption data for rats fed the purified lactose ration at the high vitamin level with butterfat and corn oil are given in greater detail in Table VI. During the first five days there was no difference in the amount of food consumed by the two groups. The group fed butterfat began to eat more than the group fed corn oil between the sixth and tenth day. After two weeks, consumption

differed markedly. If palatability were a factor in determining the growth response of rats to these two fats as contended by Deuel and Movitt (6), a difference in consumption should be apparent from the start of the experiment.

No gross pathology of the internal organs was observed in rats fed any of the diets described. The fresh weight of the cecum of animals fed these rations, characterized by 48% of carbohydrate and 28% of either fat, was usually in the range of 2 to 3 g. at the end of an experiment with the exception of rats on lactose rations. Ceca ranged from 3.5 to 10 or more g. with either  $\alpha$ - or  $\beta$ -lactose in the diet. Considerable water was present. It was noticed that the cecal contents of rats fed the butterfat-lactose rations were light yellow in color and usually quite free of gas. In contrast, the cecal contents of the corn oil-lactose group were gray and gas occluded with an odor of putrification.  $H_2S$  was easily driven off and detected with lead acetate. This difference in color was reflected in the feces of the animals in these respective groups, *i.e.*, those from the butterfat-lactose animals were light colored, while those from the corn oil-lactose group were black. Cream colored feces were sometimes noted when butterfat was fed with any carbohydrate.

The general appearance of rats fed corn oil with lactose was poor at all vitamin levels. This was also true of rats fed starch, dextrin, dextri-maltose, fructose-glucose mixture, and sucrose with a medium level of vitamins and occasionally of rats fed the high vitamin corn oil-sucrose ration. The chief gross symptoms were "bloody" nose and head, poor fur, and extensive symmetrical abdominal alopecia. Diarrhea was prevalent among rats fed lactose with corn oil, especially during the first 3 weeks, but was absent or much less evident among the rats fed the ration with butterfat. A mild form of dermatitis of the paws was found only among animals fed the lactose ration with corn oil.

The *in vitro* studies showed that the addition of the fatty acids of either fat had little effect on the growth of *S. lactis* up to levels of 2.0 mg. per tube. Complete inhibition of growth apparently resulted from the addition of 4.0 mg. The growth of *L. casei* was not inhibited with levels of the fatty acids of butter fat up to 0.5 mg. per tube, but levels of 1.0 and 2.0 mg. showed increasing inhibition, which became complete at levels of 4.0 mg. In contrast, the free fatty acids of corn oil inhibited the growth of *L. casei* at all levels tested.

### DISCUSSION

The data indicate that the apparent requirement of the rat for vitamins of the B-complex may be altered by a change in the kind of dietary fat, depending upon the kind of carbohydrate used in the ration. If this phenomenon were a function of a difference in metabolism of the fats studied, it would be expected to be manifest at certain vitamin levels irrespective of the carbohydrate fed, but this was not the case. Apparently the flora existing by virtue of the diets containing sucrose, a fructose-glucose mixture, starch, dextrin, dextri-maltose, and lactose was labile to the kind of fat fed. The data obtained on the sucrose ration will serve to illustrate this point. The groups of rats which received butterfat gained 185 g. at the medium level of vitamins, 189 g. at the high level, and 209 g. in 6 weeks at the high level of vitamins plus 1 per cent of whole liver concentrate. In contrast, rats fed the above rations with the butterfat replaced by corn oil grew 148, 166, and 203 g., respectively. At the lowest (medium) level of vitamins fed, growth of rats receiving butterfat surpassed those on corn oil by an average of 37 g. in 6 weeks. The superiority of butterfat was non-existent (6 g.) at the high level of vitamins plus whole liver powder. The poor growth of the group receiving the corn oil ration at the medium level of water-soluble vitamins is a reflection of a reduced quantity of vitamins available to the animal from the intestinal flora, since the addition of more synthetic B-vitamins plus liver concentrate allowed these animals to grow at a rate equal to that achieved by those fed butterfat. The B-vitamin content of butterfat is negligible.

The *in vitro* experiment which showed the effect of the fatty acids in butter fat and corn oil on the growth of bacteria was described solely as an analogy to illustrate the probable mode of action of the fats in the interplay of fat, carbohydrate, and B-vitamins in the nutrition of the rat. No implied significance of either organism *in vivo* is intended. If a portion of the vitamin synthesizers or utilizers in the tract were similarly inhibited or allowed to grow, significant differences in the kind and amount of vitamins available to the host would be possible.

The qualitative and quantitative nature of the underlying deficiencies demonstrated in rats fed corn oil in combination with various carbohydrates may not be the same. For example, the riboflavin

produced in the intestine of rats was shown to be much greater on diets high in lactose or dextrin as compared to sucrose (18). The observation that supplements of high levels of thiamine, riboflavin, pyridoxin, pantothenic acid, and choline, in addition to *p*-aminobenzoic acid, inositol, and nicotinic acid improved the growth of rats fed certain corn oil rations indicates that one or more of these vitamins may be involved. Biotin and unknown vitamins are not ruled out since the inclusion of a liver concentrate in the sucrose, dextro-maltose, and lactose diets was more effective than high B-vitamins alone in equalizing the response of rats to the two fats. Furthermore, an interplay among the vitamins may be operative. McIntire, *et al.* (19) found that supplements of *p*-aminobenzoic acid and inositol stimulated the growth of rats fed purified diets low in thiamine.

In these experiments, the kind of fat had little effect on the growth of rats fed rations which contained glucose or the galactose-glucose mixture. Possibly in the case of these two carbohydrates, which Cori and Cori (20) have found to be most rapidly absorbed, the flora was largely determined by material other than carbohydrate (protein, salts, etc.) and changes in the kind of fat had a negligible effect on this particular flora and its ability to supply the known and unknown vitamins.

No generalization can be made concerning the effect of the mono-, di-, or polysaccharides in the diet on the growth response of the rats. Changes in the kind of dietary fat and level of vitamins caused an equivalent response in the growth of rats fed diets which contained either sucrose or the glucose-fructose mixture. At defined levels of vitamins, the galactose-glucose diets were superior to the lactose diets in growth promoting value, and only in the case of the latter diets was growth affected by a change in the kind of dietary fat. Thus, the depressed rate of growth of rats fed purified diets containing lactose cannot be attributed to the galactose portion of the molecule.

Lactose as found in milk is known to be an entirely adequate carbohydrate for nutrition. However, in contrast to other common dietary carbohydrates, the growth of rats fed lactose in certain purified rations was found to be inferior. Ershoff and Deuel (21) described rations on which rats failed to survive when lactose was substituted for other carbohydrates. From a number of possible explanations, two deserve special consideration: (1) The flora, as determined by purified rations containing large amounts of lactose, may be so com-

pletely altered that dietary essentials normally furnished by the intestinal flora, or supplied by natural food in the case of milk, are not available to the rat (9). A decrease in the bacteria classified as vitamin synthesizers or an increase in the number of vitamin utilizers, or both, may be responsible. (2) A slow rate of digestion of lactose to its component monosaccharides would also account for the conditions observed in rats fed the purified diets containing lactose. At autopsy, the intestinal tract of rats fed lactose diets is invariably distended due to the presence of the disaccharide which, because of the high osmotic pressure developed, retains considerable quantities of water in the tract. Furthermore, an energy handicap may thus be affected as compared to the readily absorbed carbohydrates, and the presence of the water might tend to inhibit proper absorption of all classes of food material. Essentially the same possibilities were considered by Ershoff and Deuel (21) in explanation of the mortality of rats fed rations which contained 73.2 per cent of lactose.

In support of the first explanation, a number of examples may be cited to show that the growth of rats fed rations containing lactose is correlated with the vitamin content of the ration rather than the level of lactose. Kemmerer, *et al.* (22) showed that a rat required about 35 days to grow 140 g. when fed whole milk alone. Only 2.25 g. of milk solids were required to produce 1 g. gain in body weight. A ration described by Geyer, *et al.* (1) was composed of 50 parts of skim milk powder as the sole source of B-vitamins, minerals, and protein, to which was added 30 parts of fat and 20 parts of lactose, thus bringing the total lactose content to about 45 per cent. Apparently the level of vitamins was critical in this ration. With butterfat, growth of rats over a 6 weeks period was 214 g. in contrast to 172 g. for rats fed the same ration containing corn oil. Rats also grew well on a ration which contained 46 per cent of lactose together with a daily supplement of 2.5 g. of raw pork liver per rat (Table IV). In six weeks, growth was only 13 g. less than that obtained on the same ration containing sucrose. The growth of rats fed various lactose rations is compared with the number of grams of lactose ingested (Table VII). The amount of ration necessary to produce one gram gain in body weight is also shown. It can be seen that there was a tendency for the rats to utilize more lactose as the vitamin level of the diet was raised. The addition of a high level of vitamins plus liver and casein to a purified ration containing lactose and only a medium level

of 5 B-vitamins nearly doubled the growth of rats and, as a corollary, less ration was required to produce one gram gain in body weight. It is apparent that rather large amounts of lactose may be well utilized if adequate amounts of B-vitamins are available. Ershoff and Deuel (21) found that of all substances tested, only liver gave some indication of reducing the mortality of rats on the high lactose diet. It should be pointed out, however, that they ignored the necessity for fat in the utilization of galactose by the rat as shown by Schantz, Elvehjem, and Hart (23). The final explanation of the peculiar nutritional properties of lactose may well be complex and involve a combination of such factors as lactase activity and intestinal vitamin balance.

TABLE VII  
*Economy of Food Utilization of Rats Fed Lactose and Butterfat in Rations Carrying Various Amounts of Vitamins*

Source of data	Whole milk	Raw liver High vitamins	Liver powder High vitamins		High vitamins		Medium vitamins	
	Kemmerer, <i>et al.</i> (23)	Expt. 106*	Expt. 119	Expts. 122 and 126**	Boutwell, <i>et al.</i> (2)	Expts. 88, 89, 93**	Expt. 110**	Expt. 125**
Ration consumed, g.		474	438	411	416	424	355	392
Lactose consumed, g.		212.6	210.2	197.2	199.7	203.5	170	188
Gain, g.	140	215	169	168	145	149	120	116
Grams of ration required to produce 1 g. gain	2.25	2.20	2.50	2.45	2.86	2.77	2.95	3.38
Number of rats	6	6	6	12	12	18	6	6

\* See Table IV.

\*\* See Table II.

Weanling rats, when fed *ad libitum* a defined ration in which several carbohydrates could be substituted at specified levels of B-vitamins, showed a superior response to butterfat in comparison to corn oil if supplemented with adequate amounts of the known fat-soluble vitamins. It was found that under many conditions butterfat and corn oil were of equal growth promoting value for the rat. This has also been shown in the extensive studies of Deuel, *et al.* (3, 4, 5, 6) and of Zialcita and Mitchell (7). However, the latter investigators, in criticizing our data, interpreted their negative results to cover all conditions under which these fats may be fed.

These experiments, showing the influence of fats on the synthesis of water-soluble vitamins in the intestine, raise many questions which

cannot be answered at the present writing. Would man subsisting on a diet low in the water-soluble vitamins be further endangered by the consumption of vegetable oils or their products as compared with the animal fats, especially butterfat? The problem is worth investigating.

### SUMMARY

1. A change in the kind of dietary fat altered the apparent requirement of the rat for vitamins of the B-complex when sucrose, a fructose-glucose mixture, starch, dextrin, dextri-maltose, or lactose was the carbohydrate in certain rations.

2. On any of the above carbohydrates, rats which received butterfat and a medium level of thiamine, riboflavin, pyridoxin, pantothenic acid, and choline grew at a faster rate than comparable rats fed corn oil. This inferiority of corn oil could be reduced on lactose rations and eliminated on all other rations by raising the level of these vitamins and adding high levels of inositol, *p*-aminobenzoic acid, and nicotinic acid plus 1 per cent of whole liver powder.

3. No difference in growth between the rats receiving butterfat and those fed corn oil was obtained at any vitamin level when glucose or a galactose-glucose mixture was the carbohydrate portion of the ration.

4. Rats receiving either of the two fats on the lactose ration grew less than animals fed similar rations containing other carbohydrates, but this inferiority decreased as the level of the water-soluble vitamins was increased. The galactose *per se* was not responsible for this retarded growth.

5. Possible explanations of the observed phenomena are briefly discussed in the text.

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# Catalase in Serum and Plasma \*†

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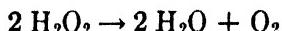
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Received March 22, 1945

## INTRODUCTION

Recently we have been engaged in the isolation and characterization of the proteins of lymph and serum from burned animals (1). In the course of these studies, a report by Jaques (2) stating that preparations of fibrinogen readily decomposed hydrogen peroxide seemed of interest. Jaques attributed the observed decomposition to unidentified groups in the fibrinogen molecule. We investigated this phenomenon and found that fibrinogen-free protein preparations from serum, plasma, and lymph also react with hydrogen peroxide.

Because of the large amount of peroxide decomposed by the protein preparations, we suspected that an enzymatic mechanism was involved. We observed that cyanide and hydroxylamine, which are known to be inhibitors for the enzymes catalase and peroxidase, completely blocked the reactions of the protein preparations. Gasometric measurements showed that the amount of oxygen liberated by the catalysis corresponded to the reaction:



and we therefore concluded that catalase was the enzyme present. In this report the presence of catalase in normal human serum and plasma is established, and the distribution of the enzyme in the fractions obtained by the separation of the plasma proteins is described.

\* This is publication No. 85 of the Robert W. Lovett Memorial for the study of crippling disease, Harvard Medical School.

† The expenses of this investigation were defrayed in large part by a grant from the Commonwealth Fund.

### METHODS AND MATERIALS

*Determination of Catalase.* The catalytic decomposition of hydrogen peroxide was followed by measuring the oxygen evolved by a manometric technique employing 7 to 15 cc. Warburg vessels with a single side-arm. The test solution was buffered at pH 6.6 to 6.7 by the addition of 0.25 M phosphate buffer; the enzyme concentration was adjusted to give a velocity constant  $K^{30^\circ}$  of 0.025 to 0.05. After temperature equilibration in a water bath for five minutes at  $30^\circ\text{C}.$ ,<sup>1</sup> the reaction was initiated by tipping 0.25 cc. of 0.038 N hydrogen peroxide into the buffered solution. The reaction was usually followed until the theoretical amount of oxygen had been evolved. The decomposition of hydrogen peroxide is approximately monomolecular during this period. The K value obtained by correcting  $K^{30^\circ}$  to standard temperature,  $0^\circ\text{C}.$ ,<sup>2</sup> and extrapolating to zero time can be employed directly as an expression of the units of catalase (4).

*Hydrogen Peroxide.* A fresh solution was prepared for each experiment from a stock solution of Perhydrol "Merck," and the peroxide content checked by iodometric titration (5). One cc. of 4 N sulfuric acid, one cc. of 60% potassium iodide, and one cc. of 1.0% ammonium molybdate were added to a sample of hydrogen peroxide and the liberated iodine determined with 0.01 N sodium thiosulfate.

*Serum, Plasma, and Protein Fractions.* Serum was obtained by centrifuging normal human blood which had been allowed to clot. For the preparation of plasma the blood was treated with potassium oxalate to prevent coagulation (2.0 mg. per 1.0 cc. of blood). Both the serum and plasma were tested immediately for the enzyme. The protein fractions used in some of the experiments were prepared from normal human plasma by the methods developed by Cohn, *et al.* (6).<sup>3</sup>

*Hemoglobin Determination.* In some experiments it seemed of interest to estimate the increase in hemoglobin due to hemolysis. The color absorption of 0.5 cc. of hemolyzed serum diluted to 10.0 cc. with 0.15 M sodium chloride was compared with that of a similarly diluted sample of normal serum obtained from the same blood. The measurements were made with a Coleman spectrophotometer using a wave length of 560 m $\mu$ . A standard curve based on the oxygen capacity of a sample of normal human blood, determined according to the method of van Slyke, was used in the calculation of the amount of hemoglobin present.

<sup>1</sup> A temperature of  $30^\circ\text{C}.$  was chosen for convenience. No appreciable loss of activity was found at this temperature when the concentration of the substrate was kept low.

<sup>2</sup>  $K^{30^\circ}$  was corrected to  $0^\circ\text{C}.$  by using the Arrhenius equation

$$\mu = 4.6 \frac{(\log K_2 - \log K_1)}{1/T_1 - 1/T_2};$$

$\mu$  = 4200 calories per g. molecule (3).

<sup>3</sup> The authors are indebted to Drs. E. J. Cohn, J. D. Ferry, and J. L. Oncley of the Department of Physical Chemistry, Harvard Medical School, for supplying some of the protein preparations used in this investigation. These were produced under contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and Harvard University.

## RESULTS

*Identification of the Enzyme as Catalase.* Hydrogen peroxide is readily decomposed by serum, plasma or protein fractions obtained from plasma. One mole of oxygen is obtained from two moles of hydrogen peroxide. A typical experiment, in which the catalysis is caused by a protein preparation (Fraction I), containing about 60 per cent fibrinogen (ratio of clottable nitrogen to total nitrogen) is shown in Fig. 1. The fact that the theoretical amount of oxygen is

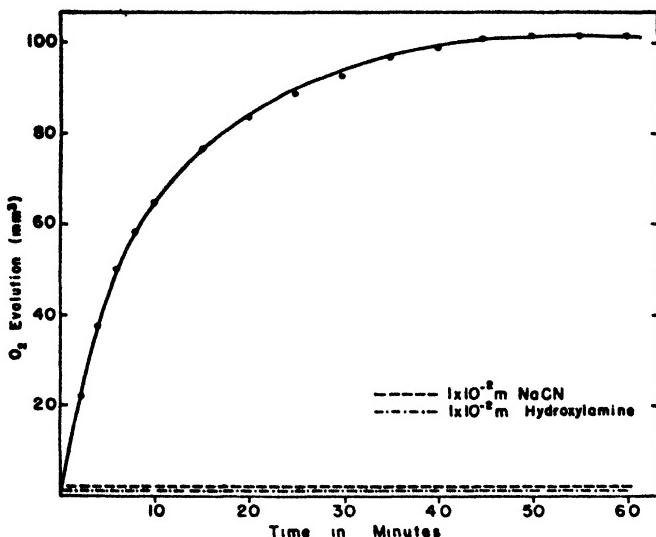


FIG. 1

Oxygen Evolution from 0.25 cc. 0.038 N  $H_2O_2$  at pH 6.7 at 30°C. is Plotted Against Time

The reaction is catalyzed by 1.44 mg. of a fibrinogen-containing preparation (Fraction I)

obtained, and the fact that the reaction is inhibited by 0.01 *M* cyanide and 0.01 *M* hydroxylamine, are considered as essential proof that catalase is present in the preparation.

*Catalase in Serum and Plasma.* Although the red cells have long been known to be a rich source of catalase, this enzyme has not been found previously in serum and plasma. Results obtained from a number of determinations using normal serum and plasma are shown in Table I.

TABLE I

*Catalase Content of Normal Human Serum and Plasma*

0.1 cc. serum or plasma was diluted to 1.5 cc. with 0.25 M phosphate buffer, pH 6.6, and 0.25 cc. 0.038 N  $\text{H}_2\text{O}_2$  was added.

Experiment No.	Material	Protein in sample mg.	$K^{30^\circ}$ **	$\frac{\text{"Kat f"} \times 100}{43,000} \times 10^{-4}$
1.	Serum	7.0	0.0586	2.85
2.	Serum	6.4	0.0425	2.35
3.	Serum	6.8	0.0546	2.76
4.	Serum	6.8	0.0590	2.90
5.*	Serum	7.6	0.0590	2.64
	Plasma	7.8	0.12	5.25
6.*	Serum	6.9	0.0416	2.06
	Plasma	7.2	0.083	3.95
7.*	Serum	7.2	0.0302	2.35
	Plasma	6.9	0.082	4.10

\* In each of the experiments 5, 6, and 7 the sera and plasma were obtained from the same blood.

\*\* K, the monomolecular reaction constant, was calculated according to the formula,

$$K = \frac{1}{t_2 - t_1} \cdot \log \frac{a - x_1}{a - x_2}$$

The monomolecular reaction constant is calculated from the readings made during the first ten minutes. In column 5 of the table the amount of the enzyme is expressed as per cent of the total protein. The equation:

$$\% \text{ catalase} = \frac{\text{"Kat f"} \times 100}{43,000} \quad (7)$$

is used for calculation.<sup>4</sup> The value 43,000 is taken for pure catalase from the report by Laskowski and Sumner (8) on crystalline erythrocyte catalase. From the table it can be seen that catalase occurs both in normal human serum and in plasma. However, the activity in plasma is about twice that found in the corresponding serum.

A possible explanation of the difference between plasma and serum is that the former contains fibrinogen, which has been considered to be responsible for the catalysis (2). A second possibility may be that

<sup>4</sup> The expression, "Kat f" =  $\frac{K^{\circ}}{\text{g. enzyme preparation/50 cc.}}$ , means catalase purity (4).

the fibrin clot removed from serum also removes the active substance. That neither of these explanations is correct is shown by the experiment reported in Table II, where the catalase activities of serum, of

TABLE II

*Catalase Content of Serum, Plasma, and Defibrinated Plasma*

0.1 cc. serum or plasma was diluted to 1.5 cc. with 0.25 M phosphate buffer, pH 6.6, and 0.25 cc. 0.038 N H<sub>2</sub>O<sub>2</sub> was added.

Material	Protein in sample mg.	K <sup>30°*</sup>	$\frac{\text{"Kat f"} \times 100}{43,000} \times 10^{-4}$
Serum	6.6	0.027	1.37
Plasma	6.4	0.047	2.4
Defibrinated plasma	6.5	0.048	2.4

\* Cf. second footnote to Table I.

plasma, and of defibrinated plasma prepared by the recalcification method of Cullen and van Slyke (9) are compared. All of these solutions were obtained from the same blood and it is apparent that defibrination of plasma is not accompanied by decrease of catalase activity. Similarly, the transformation of the fibrinogen of Fraction I, where it is the major protein constituent, into fibrin by the addition of thrombin prepared from human plasma (Fraction III-1-2) also does not result in significant loss of the enzyme, as shown in Table III. The thrombin solution itself was free of activity and tests of the resulting fibrin clot showed that it did not have catalase action.

TABLE III

*Catalase Activity of Fraction I, Before and After Clotting*

Preparation No.	Material tested	Protein in sample mg.	K <sup>30°*</sup>
1	Fraction I	3.36	0.042
	Thrombin	0.4	0
	Fibrin clot	2.02	0
	Fraction I, after removal of clot	1.34	0.040
2	Fraction I	2.55	0.052
	Thrombin	0.2	0
	Fibrin clot	1.53	0
	Fraction I, after removal of clot	1.02	0.050

\* Cf. second footnote to Table I.

These experiments indicate that the enzyme is not associated with the proteins involved in the clotting mechanism.

Since it is known that red cells contain catalase, we were led to suspect that the difference between plasma and serum shown in Table I might be traced to different amounts of red-cell destruction obtained by the techniques employed in the preparation of the respective samples. Thus, the use of solid potassium oxalate as anti-coagulant in preparing the plasma might have caused slight hemolysis.

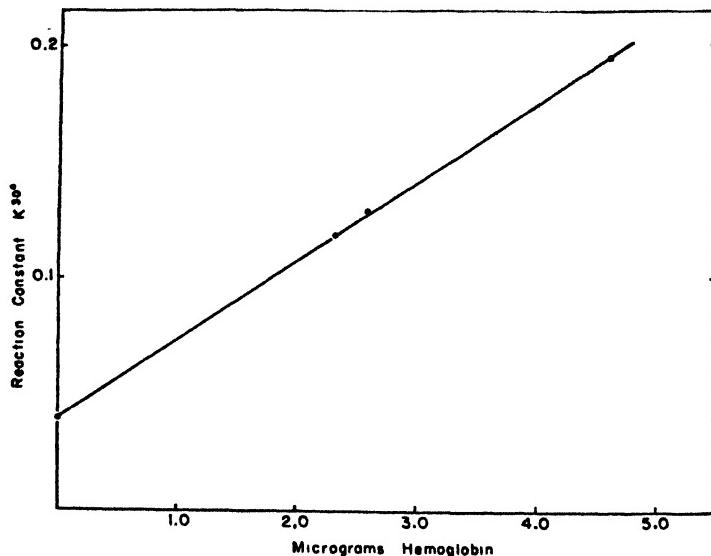


FIG. 2

Relation of Catalase Content to the Hemoglobin Concentration of Different Samples of Slightly Hemolyzed Serum Obtained from the Same Blood

Therefore, experiments were made to test the relationship between hemolysis and catalase content.

Samples of slightly hemolyzed serum were obtained by mechanical injury of the red cells. The catalase activity and hemoglobin concentration were measured and compared with normal serum from the same blood. In Fig. 2 the enzyme activity was plotted against the hemoglobin content. A straight line was obtained. Since crystallized hemoglobin was found to be inactive this proportionality must be

attributed to a concomitant release of catalase and hemoglobin from broken red cells.

*Distribution of the Enzyme in Plasma Fractions.* When it was found that the decomposition of hydrogen peroxide is not caused by the proteins involved in the clotting mechanism, it was of interest to test all the plasma fractions for their catalase activity. First, the activities of plasma which had been kept frozen and of a sample dried from the frozen state were compared. It was found that the activity of the dried preparation had decreased to 44% of its original value during the drying process. This is in accord with some recent observations reported by Dounce and Howland for crystalline beef liver catalase (10). Various protein fractions obtained by methods recently described were then tested. The enzyme was demonstrated in Fractions I, II + III, and IV, which contain the globulins of the plasma.<sup>5</sup> The "Kat f" of Fraction I exceeds the values found for the remaining fractions but represents only 20% of the total activity. Some loss of activity was shown to have taken place during the procedure involving the separation of Fraction II + III. The low content found in Fraction IV might be the result of further inactivation. Because of the many factors involved, it is difficult to give a more accurate account of the distribution of the enzyme. However, these experiments, as well as some additional observations made on fractions obtained by ammonium sulfate precipitation, indicate that the enzyme activity is associated with the globulins of plasma and is not found in the albumin-containing fraction. The failure to isolate the enzyme in one of the globulin fractions is only to be expected in view of its concentration, which is so low that it can only be detected through its biological activity.

#### DISCUSSION

The decomposition of hydrogen peroxide by normal human serum, plasma and plasma fractions appears to be an enzyme reaction due to traces of catalase. The amount of the enzyme is in the range of 15 to 19 µg. in 100 cc. of serum.

Considering the great abundance of catalase in erythrocytes, it appears most likely that even the slightest injury of red cells may

<sup>5</sup> The main constituents of the fractions according to electrophoretic analysis are: Fraction I = fibrinogen,  $\beta$ - and  $\gamma$ -globulins, traces of albumin; Fraction II + III =  $\gamma$ -,  $\beta$ -,  $\alpha$ -globulins, traces of albumin; Fraction IV =  $\alpha$ -,  $\beta$ -globulins (6).

cause a measurable appearance of the enzyme in serum. This may not, however, be the only source of catalase, since the enzyme is generally present in body cells. Our experiments with artificially hemolyzed serum demonstrate an apparent relationship between the release of hemoglobin and of catalase. Nevertheless, for normal sera rather uniform data are obtained. This indicates that under reasonably standardized conditions gross changes in catalase may be significant. The question as to whether catalase activity could be used as a test for traces of hemolysis or of the fragility of red cells in pathological cases is now under investigation.

### SUMMARY

Normal human sera and plasma are found to decompose hydrogen peroxide. The reaction is completely inhibited by cyanide and hydroxylamine. The amounts of oxygen produced are equivalent to the chemical disappearance of hydrogen peroxide. From these facts it is concluded that catalase is present in serum and plasma.

On fractionation of the plasma proteins, the enzyme activity is found in the globulin fractions.

The origin of serum and plasma catalase is discussed.

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# The Function of Pyridoxin Derivatives: A Comparison of Natural and Synthetic Codecarboxylase

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Received March 29, 1945

## INTRODUCTION

The purpose of this paper is to provide further evidence, through the isolation and resolution of the enzyme, that a derivative of pyridoxal is the coenzyme of tyrosine decarboxylase and to compare the properties of the natural coenzyme described by Gale and Epps (1) with that prepared from pyridoxal (2). An assay system is described which permits one to distinguish between, and to quantitatively estimate, pyridoxal and the coenzyme.

Work on the isolation and resolution of tyrosine decarboxylase from *Streptococcus faecalis* R was in progress when the paper of Epps (3) on the preparation of the enzyme from a similar streptococcus appeared. Although we had used somewhat different methods from those employed by Epps (3), the properties described are so similar that it seems quite certain we are dealing with the same complex.

Gale and Epps (1) have applied the term "codecarboxylase" to the coenzyme which they isolated from yeast and showed to function in the decarboxylation of tyrosine and lysine (4). They gave no indication that the coenzyme was a pyridoxin derivative, whereas previous publications from this laboratory have shown that members of the vitamin B<sub>6</sub> group (5, 6), especially pyridoxal (7), function in the tyrosine decarboxylase system. Thus it became important to compare the codecarboxylase of Gale and Epps (1) with the coenzyme derived from pyridoxal (2) to determine whether or not the properties are sufficiently similar to indicate their identity. The term "codecarboxylase" (1) is used in the present paper to include the synthetic substances prepared from pyridoxal (2) in addition to the natural substance.

Gale and Epps (1) reported that the yeast codecarboxylase is free from phosphorus, whereas the procedures we employ for the preparation of the codecarboxylase from pyridoxal certainly imply that the coenzyme is phosphorylated. Synthesis from pyridoxal can be accomplished either biologically, using adenosinetriphosphate

(ATP), or chemically, by using phosphorylating agents. However, as the structure is unknown, it is possible that the synthetic coenzyme does not contain phosphorus. The determination of the presence of phosphorus in the synthetic coenzyme is complicated by the large excess of inorganic phosphate which results from the method of synthesis. Further studies of the properties of the coenzyme from pyridoxal, the presence of phosphorus in the molecule, and improved methods of synthesis will be described elsewhere.

## METHODS

Conventional Warburg methods were employed throughout. Tyrosine decarboxylation was followed by measuring the evolution of carbon dioxide at pH 5.2 in 0.075 M phthalate buffer or at pH 5.5 in 0.07 M acetate buffer. A temperature of 28°C. was used because higher temperatures cause an inactivation of the enzyme system. The protein content of the enzyme preparations was determined by the method of Robinson and Hogden (8).

The culture used throughout this study was *Streptococcus faecalis*, strain R (American Type Culture Collection No. 8043—previously under the name of *Streptococcus lactis*).

### Tyrosine Decarboxylase Enzyme Systems

*Enzyme from Vitamin B<sub>6</sub>-Deficient Cells.* It has previously been demonstrated that *Streptococcus faecalis* cells grown in the absence of pyridoxin derivatives are unable to decarboxylate tyrosine (5, 9) and that the apoenzyme may be prepared from these cells (2). Good growth of the R strain in the absence of the vitamin B<sub>6</sub> group is based on Snell and Guirard's (10) discovery that alanine will replace these substances for growth. A detailed study of the factors which affect the production and activity of coenzyme-deficient cells appears in the report of Bellamy and Gunsalus (11). For the cell preparations employed in this study the medium shown in Table I was employed.

The cells were harvested by centrifugation after 18 to 20 hours' incubation at 37°C.; washed with saline, suspended in distilled water, and dried *in vacuo* over drierite. The resulting dry cell powder is stable for long periods if kept dry, and is capable of decarboxylating tyrosine when natural or synthetic codecarboxylase (or pyridoxal plus ATP) is added. This type of enzyme system is also used in the assay method for coenzyme.

*Enzyme Isolated from Cells Grown in a Complex Medium.* Cells harvested from complex media and vacuum- or acetone-dried preparations from such cells are capable of decarboxylating tyrosine without the addition of the coenzyme. In order to demonstrate the function of a coenzyme with this type of system, it is necessary to separate the enzyme from the cells and to remove the coenzyme from the complex.

As the publication of Epps (3) describes a method for isolating the cell-free enzyme, a detailed description of our procedure is unnecessary. The essential details summarizing this work are given in Fig. 1. The culture was incubated for 18 hours in a medium containing 1% each of tryptone, yeast extract, and glucose, and 0.5% of

$K_2HPO_4$  (9), and the cells harvested with a Sharples supercentrifuge. After one washing with saline, they were suspended in water, and dried *in vacuo* over drierite.

The enzyme activity per milligram of dry cells was less than that reported by Epps (0.08 units/mg. as compared to 0.53 units/mg. obtained by Epps). One unit of enzyme is the amount required to release 100  $\mu$ l. of carbon dioxide from tyrosine in five minutes under the conditions outlined above. Although the lower activity is partially a characteristic of the culture, the quantity of enzyme per cell may be

TABLE I  
*Defined Medium for the Growth of Pyridoxal Deficient Cells*

	<i>per liter</i>
Hydrolyzed casein, vitamin-free	10 g.
Glucose	10 g.
$K_2HPO_4$	5 g.
Sodium acetate	2 g.
Salts B*	5 ml.
Alanine	200 mg.
Cystine hydrochloride	200 mg.
Tryptophan	100 mg.
Sodium thioglycolate	50 mg.
Adenine sulfate	25 mg.
Guanine hydrochloride	5 mg.
Uracil	5 mg.
Nicotinic acid	5.0 mg.
Riboflavin	1.0 mg.
Calcium pantothenate	1.0 mg.
Thiamine chloride	.7 mg.
S. L. R. factor†	2.5 $\mu$ g.
Biotin	1.0 $\mu$ g.

\* Salts B, per 250 ml.  $H_2O$ : 10 g.  $MgSO_4 \cdot 7H_2O$ ; 0.5 g. each  $NaCl$ ;  $FeSO_4 \cdot 7H_2O$ ;  $MnSO_4 \cdot 4H_2O$ .

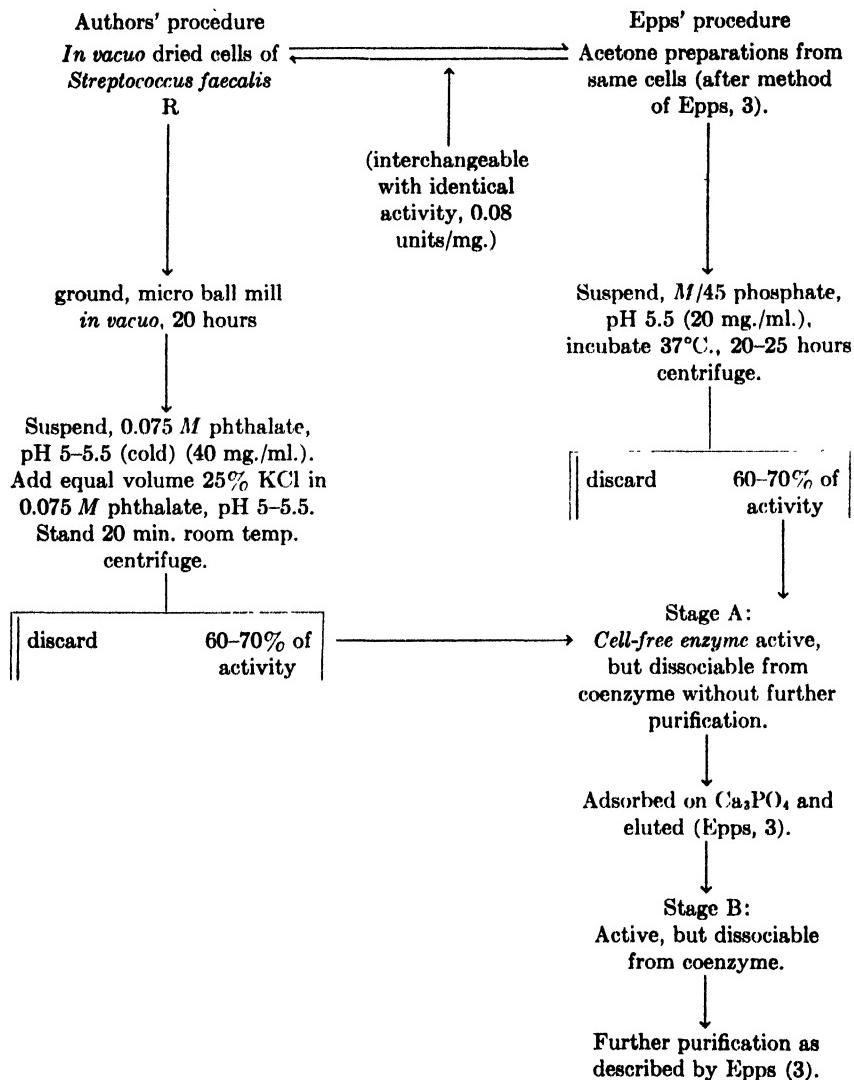
† We are indebted to the Research Laboratories of Merck & Co., Inc. for the supply of the *Streptococcus lactis* R factor.

increased by altering the cultural conditions; for example, by a reduction of the buffer content of the medium (9).

Adequate separation of the codecarboxylase for studies of its substitution could be obtained by purification of the enzyme through stage B of the procedure of Epps (3) (Fig. 1). Therefore, further purification was not attempted. The enzyme with this degree of purity was desirable as it also contained the enzymes which convert pyridoxal plus ATP into the coenzyme. This was especially valuable as a large share of the present work was completed before relatively pure samples of synthetic codecarboxylase were available.

FIG. 1

## Preparation of Cell-Free Tyrosine Decarboxylase



*Preparation of Codecarboxylase*

*Isolation of Codecarboxylase from Yeast.* The natural coenzyme was obtained from yeast by purification through stage 2 of the procedure of Gale and Epps (1). This involves extraction of dried brewers' yeast with 0.75% Ba(OH)<sub>2</sub>·8H<sub>2</sub>O; and removal of the barium as the sulfate by acidification to pH 3.0. The proteins were precipitated with two volumes of methanol, and the coenzyme precipitated from the supernatant solution by the addition of barium acetate. The coenzyme was recovered from the methanol-barium precipitate by elution with sulfuric acid. The extract was neutralized to pH 7.0 and held in the refrigerator; before use it was adjusted to pH 5.5. Gale and Epps report that this treatment results in a purification of about thirty times without great loss of the coenzyme. To distinguish this preparation from other materials with codecarboxylase activity, it is designated in this paper as the "natural coenzyme."

*Biological Preparation of Codecarboxylase from Pyridoxal.<sup>1</sup>* Two types of systems were used for the conversion of pyridoxal into the coenzyme in the presence of ATP. These were: first, the dried preparations from cells grown on a vitamin B<sub>6</sub> deficient medium; and, second, the enzyme extracted from cells grown in complex media and partially resolved into apo- and co- enzyme. The coenzyme was prepared by adding pyridoxal and ATP to the enzyme and incubating a few minutes before the addition of substrate. This type of preparation is referred to as "pyridoxal coenzyme."

*Chemical Conversion of Pyridoxal to Codecarboxylase.* Two chemical methods for the preparation of codecarboxylase from pyridoxal have already been suggested (2). These, however, yielded only traces of active material and were rather unreliable.

The synthetic coenzyme used in this work was prepared from pyridoxal by a modification of the Zeile and Fawaz (12) method for synthesis of phosphocreatine. This was accomplished by dissolving 21.8 mg. of pyridoxal hydrochloride in 2.2 ml. of water and adding 0.02 ml. of 1:1 NaOH. The solution was chilled in an ice bath and 0.1 ml. of POCl<sub>3</sub> plus 0.4 ml. of 1:1 NaOH added. The mixture solidified in the ice bath and was removed and warmed until the precipitate dissolved, then chilled again. The addition of the same amounts of POCl<sub>3</sub> and NaOH was repeated four times, with warming between each addition. After the last treatment, the solution was chilled until small crystals formed throughout the tube, and was then

<sup>1</sup> We wish to thank Merck & Co., Inc., Research Laboratories, for the pyridoxal used in these studies.

centrifuged while cold. The precipitate (presumably  $\text{Na}_3\text{PO}_4$ ) contained only a small portion of the activity and was therefore discarded. The supernatant was made faintly acid to phenolphthalein and diluted to 25 ml. An assay for codecarboxylase showed 36  $\mu\text{g}$ . per ml., based on the weight of pyridoxal which would give equal coenzyme activity (see assay procedure). If all the coenzyme formed appeared in this fraction, the yield was about 5% of the pyridoxal used. A portion of this preparation was partially purified by barium fractionation as follows: 10 ml. were cooled, acidified to pH 3.0 with  $\text{HCl}$ , and barium acetate added. The precipitate which formed was not removed until after neutralization to pH 8.2, which caused further precipitation. The precipitate was removed by centrifugation, the supernatant poured into five volumes of alcohol, readjusted to pH 8 and chilled. The alcohol precipitable fraction was collected, dissolved in dilute acid and the barium removed with sulfate. This fraction (5.2 ml.) contained about 40% of the coenzyme taken for purification. Analysis showed 27.6  $\mu\text{g}$ . coenzyme activity (in terms of pyridoxal) and 88.5  $\mu\text{g}$ . organic phosphate per ml. However, 72% of the organic phosphorus was hydrolyzed by seven minutes heating at 100°C. in  $N \text{ HCl}$ , without decrease in the codecarboxylase activity. The 24.8  $\mu\text{g}$ . of organic phosphorus which remained per ml. was about 50% of that required for one phosphate per molecule of pyridoxal present in the form of coenzyme. It is not yet certain whether this organic phosphorus is associated with the coenzyme or with some other organic phosphorus compound which may be present in the fraction.

Although this preparation is not pure, 0.02 ml. (0.5  $\mu\text{g}$ . pyridoxal equivalents) will saturate the enzyme systems used. The sample is relatively free of pyridoxal, as indicated by a lack of stimulation of tyrosine decarboxylase on the addition of ATP. Unless otherwise stated, this preparation was used throughout the work as a source of "synthetic codecarboxylase."

#### *Quantitative Estimation of Pyridoxal and Codecarboxylase*

For studies of the synthesis, purification, and destruction of codecarboxylase from either natural or synthetic sources, an assay procedure capable of estimating both pyridoxal and the coenzyme is necessary. Cells grown in a vitamin  $B_6$ -deficient medium and dried *in vacuo* as previously reported (11) are suitable for this purpose. The dried cells are stable for months and yield reproducible assays.

Codecarboxylase (natural or synthetic) activates the decarboxylation of tyrosine by these preparations whereas pyridoxal is active only in the presence of ATP—under which condition it is converted into the coenzyme.

The assay procedure is as follows: To each Warburg flask is added, in the side arm, 0.5 ml. of 0.03  $M$  tyrosine; and in the main compartment, 0.4 ml. (1 mg.) of dried-cell preparation, 1.0 ml. of 0.2  $M$  acetate buffer at pH 5.5, the sample adjusted to pH 5.5, and sufficient water to bring the fluid volume to 3.0 ml. For the assay of pyridoxal 1 mg. of ATP (in 0.1 ml.) is also added. The flasks are shaken at 28°C.

for ten minutes and readings begun. As soon as these show no change over a five-minute interval, the tyrosine is tipped into the main compartment and the  $\text{CO}_2$  evolved during five successive five-minute intervals recorded. The average  $\mu\text{l}$ .  $\text{CO}_2$  evolved per five minutes multiplied by twelve equals the  $Q_{\text{CO}_2}$ . For the assay of pyridoxal it is necessary to incubate the pyridoxal and the ATP in the presence of the cell preparation for at least ten minutes before the tyrosine is added; this will be discussed more fully later. ATP is supplied at the 1 mg. level, but concentrations as low as 10  $\mu\text{g}$ . will sustain the tyrosine decarboxylation in proportion to the pyridoxal present. For convenience in handling the enzyme preparation, 25 mg. quantities of the vacuum-dried powder are weighed out and are suspended as needed in 10 ml. of 0.1 M acetate buffer at pH 5.5. As suggested above 0.4 ml. of this suspension contains 1 mg. of dried cells, which is a convenient quantity for an assay. The enzyme suspension may be prepared in the morning and used throughout the day with satisfactory results if it is kept cool.

The standard curve for the assay of codecarboxylase or pyridoxal is based on pyridoxal in the presence of excess ATP. This is necessary for the present because pyridoxal is the only active compound available in pure form. The use of a pyridoxal standard introduces the assumption that both natural and synthetic codecarboxylase have the same activity as pyridoxal plus ATP. The data available at present are compatible with this assumption. Standards for the assay, given in Table II, can be plotted directly and the quantity of codecarboxylase (or codecarboxylase precursor in the presence of ATP) in an unknown sample approximated from the curve.

Estimation of coenzyme is more accurate, however, if the data are plotted in one of the linear forms of the equation suggested by Line-

TABLE II  
*Rate of Tyrosine Decarboxylation with Varied Levels of Pyridoxal*

Pyridoxal $\mu\text{g. per flask}^*$	$Q_{\text{CO}_2}$	Remarks
0.000	16.8	$K = 0.149 \mu\text{g. per 3 ml.}$
0.029	63.6	
0.059	79.0	$= 2.98 \times 10^{-7} \text{ moles per liter}$
0.088	115.0	
0.142	146.0	
0.212	175.0	$V_{\text{max}} = 476$
0.294	278.0	
0.588	343.0	
0.875	348.0	
1.160	378.0	
2.94	428.0	

\* With 1 mg. ATP per flask.

weaver and Burk (13). The dissociation of the enzyme-coenzyme complex can be calculated on the assumption that only the combined coenzyme possesses catalytic activity, and that the amount of combined coenzyme is small in proportion to the total amount present.

The equation for the dissociation constant of the complex is as follows:

$$K = \frac{(\text{coenzyme})(\text{uncombined protein})}{(\text{coenzyme protein})} = \frac{(c)(V_{\max.} - v)}{v} \quad (1)$$

where  $V_{\max.}$  is the maximum velocity of reaction for the system studied,  $c$  is the concentration of coenzyme supplied (in this case the amount of pyridoxal which is assumed to be quantitatively transformed to coenzyme in the presence of ATP), and  $v$  is the rate of reaction corresponding to a given concentration of coenzyme. When equation 1 is rearranged into one of the linear forms (13) as:

$$1/v = 1/c K/V_{\max.} + 1/V_{\max.} \quad (2)$$

and  $1/v$  plotted against  $1/c$ , one obtains a straight line from which it is possible to determine the coenzyme content of unknown samples. The most accurate range for assay purposes is from 0.05 to 0.5  $\mu\text{g}$ . of pyridoxal or the equivalent amount of codecarboxylase. The data in Table II give a  $V_{\max.} = 476$  and a  $K = 0.149 \mu\text{g}/3 \text{ ml.}$ , or  $2.98 \times 10^{-7}$  moles per liter. The latter is in remarkable agreement with the value of  $3 \times 10^{-7}$  determined by Gunsalus and Bellamy (7) using the living intact cell.

With known samples the precision of the method is about 10%, or approximately the same accuracy as the majority of the biological assay procedures. This serves to determine the recovery of codecar-

TABLE III  
*Specificity of Assay*

	Level supplied $\mu\text{g.}$	without ATP	$Q_{\text{CO}_2}$ with 1 mg. ATP
None		11.3*	10.5*
Pyridoxin†	10	10.9	11.3
Pyridoxamine†	30-300	—	13.1
4-lactone ( $\beta$ pyracin)†	10	11.4	11.4
Natural coenzyme	(0.1 ml.) 0.07	85.4	89.0‡
Synthetic coenzyme	(0.01 ml.) 0.2	259.	259.0‡
Pyridoxal	0.32	12.9	296.4

\* This value varies from 0.0 to 16.8 largely because of limitation in the manometric measurements. The value of 16.8 corresponds to 1.4  $\mu\text{l.}$  per five minutes.

† Kindly supplied by the Research Laboratories of Merck & Co., Inc.

‡ Occasional inhibition (as great as 40%) is observed when ATP is added to either the natural or synthetic coenzyme.

boxylase from natural sources, or to check the efficiency of synthesis. The method shows a marked specificity for pyridoxal plus ATP, or for codecarboxylase, as is indicated by the lack of response to other pyridoxin analogs (Table III).

#### *Properties Relating the Natural Codecarboxylase to Pyridoxal*

The data of Table III show that the three types of codecarboxylase preparations have coenzyme activity in the system prepared from

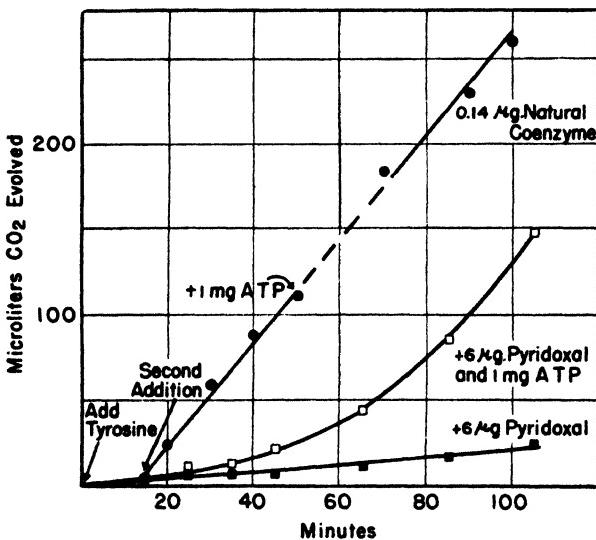


FIG. 2

#### Evidence of Coenzyme Nature of Natural Codecarboxylase

Flasks contain: 1.0 ml. 0.075 M phthalate buffer pH 5.0,

5 mg. dried preparation grown without pyridoxal and water to make volume including contents of side arms 3.0 ml.

Side arm 1: 0.5 ml. M/30 tyrosine, tipped at zero time

Side arm 2: coenzyme or pyridoxal.

deficient cells. This indicates either that the natural codecarboxylase contains pyridoxal and ATP, or that it contains the preformed coenzyme. One may decide between these two possibilities by the difference in behavior of the enzyme system in the presence of coenzyme and in the presence of pyridoxal plus ATP. If tyrosine is added to the assay system before pyridoxal (and ATP), a gradually increasing

rate of tyrosine decarboxylation is observed (Fig. 2), due to the synthesis of the coenzyme. The rate of coenzyme synthesis is apparently decreased by the tyrosine because in its absence ATP converts pyridoxal into codecarboxylase within ten minutes. That the activity of the natural preparation is due to preformed codecarboxylase is shown by the immediate evolution of  $\text{CO}_2$  at the maximal rate (see Fig. 2). This is to be expected since the isolation method (1) would almost certainly exclude ATP.

The natural codecarboxylase is, as reported by Gale and Epps (1), sensitive to acid. Even in the refrigerator at pH 5, there is a gradual loss in activity of the natural preparation (Table IV). The addition

TABLE IV  
*Loss of Activity of Natural Codecarboxylase on Storage at 0–5°C. and pH 5*

Days storage 0–5°C., pH 5	Per cent of original activity remaining	Activity remaining in the presence of ATP
0	100	—
1	98	—
5	83	—
11	32	54
16	30	37

of the aged coenzyme preparation to the assay system with ATP—thus providing an estimation of pyridoxal—shows that something which acts like pyridoxal has been produced during the inactivation of the natural codecarboxylase by storage. ATP does not entirely restore the coenzyme activity so that either materials other than pyridoxal are formed or the pyridoxal itself has decomposed.

Natural codecarboxylase preparations also lose activity when held in the refrigerator at pH 7. In this case virtually full activity is restored upon incubation with ATP in the presence of the assay system. Therefore, it appears that at pH 7.0 the coenzyme is split to yield pyridoxal which does not undergo further change. The natural codecarboxylase preparation was held in the refrigerator for 18 days at pH 7. During this time there was a loss in activity which could be restored by ATP (see Fig. 3). The natural codecarboxylase is also destroyed by acid hydrolysis. Gale and Epps (1) reported that their preparation loses 64% of its activity by boiling for one hour in  $N/10$  sulfuric acid. As pyridoxal is stable to this treatment, it should be possible to destroy the activity of the natural codecarboxylase by acid hydrolysis and to restore it with ATP, if the codecarboxy-

lase is hydrolyzed to yield pyridoxal. Upon acid hydrolysis ( $N/10$  HCl, 100°C., 60 minutes), 75% of the codecarboxylase activity was lost, all of which was restored upon the addition of ATP in the presence of the assay system (see Fig. 3). It thus appears that the natural codecarboxylase is split either by storage or by acid hydrolysis into materials which by themselves are inactive, but which are resynthesized to codecarboxylase in the presence of ATP. Inasmuch as pyri-

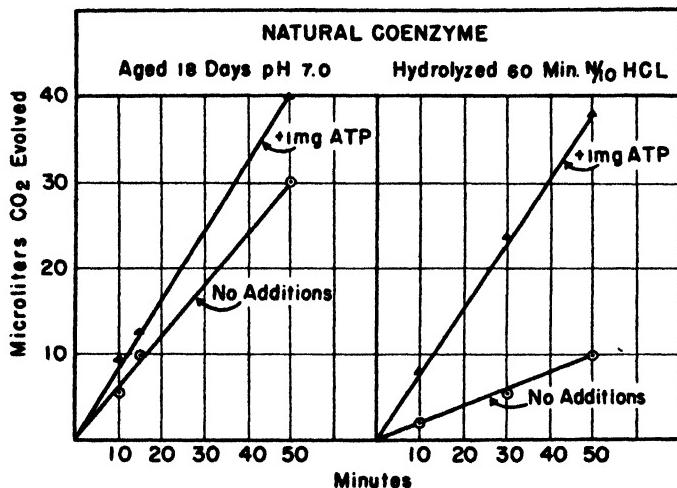


FIG. 3

Destruction of the Natural Coenzyme and the Restoration of Activity with ATP  
Flasks contain: 1 ml. 0.075 M phthalate buffer pH 5.2,

3 mg. dried-cell preparation, grown without pyridoxal; coenzyme preparation and water to total volume 3.0 ml., including side arm.

Side arm 1: 0.5 ml.  $M/30$  tyrosine, tipped at zero time.

doxal is the only known material which acts with the assay system in this manner, this evidence may be taken to indicate that pyridoxal is a component of the natural codecarboxylase. The data above do not constitute proof that the material which results from acid hydrolysis of the codecarboxylase is pyridoxal, but only that it acts like pyridoxal in the biological assay system. Whether the natural and synthetic codecarboxylase preparations possess identical structure is not known but both are active in the tyrosine decarboxylase enzyme system and both appear to be derivatives of pyridoxal.

If the natural codecarboxylase has the same activity per milligram of carbon as does pyridoxal in the presence of ATP, the purity of the Gale and Epps (1) preparation can be estimated from the assay procedure. Gale and Epps' (1) coenzyme unit is based upon the increase in rate of  $\text{CO}_2$  evolution when codecarboxylase is added to the apoenzyme. This is essentially the type of assay procedure used in the present work. The coenzyme unit (P) was defined as:  $\mu\text{l. CO}_2$  evolved per hour per mg. carbon added in coenzyme preparation. Based on pyridoxal this would be:

$$P = \frac{Q_{\text{CO}_2}(\text{pyridoxal} + \text{ATP}) - Q_{\text{CO}_2}(\text{pyridoxal, or no additions})}{\text{mg. carbon in pyridoxal used}}.$$

Over the range of 0.06 to 0.29  $\mu\text{g.}$  of pyridoxal per flask—which would very likely correspond with the range used by Gale and Epps (1), (cf. Table II) — the P value for pyridoxal is  $2.47 \times 10^6$  units per mg. of carbon. The purest preparation described by Gale and Epps had a P value of  $1.56 \times 10^6$ . If the above comparison is valid, 63% of the carbon in their preparation was codecarboxylase. This is certainly a remarkable degree of purification, especially when one considers the non-specific character of many of the fractionations which it was necessary to employ.

A cell-free enzyme may also be prepared from the cells grown in the absence of pyridoxal. However, the methods employed are slightly different from those illustrated in Fig. 1. Both the grinding and the autolysis procedure tend to destroy the apoenzyme. By incubating the dried preparation in M/5 acetate at pH 5.5 at 0°C. for ten days (2.5 mg. of preparation per ml.) an almost water-clear enzyme solution may be obtained. This still contains the enzymes capable of converting ATP and pyridoxal into the coenzyme. The activity of this apoenzyme based on  $Q_{\text{CO}_2}$  (protein) was: without additions, 43.2; with 0.6  $\mu\text{g.}$  of pyridoxal plus one mg. of ATP, 600; with 0.5 ml. (0.35  $\mu\text{g.}$ ) of natural coenzyme, 480; and with 0.01 ml. (0.2  $\mu\text{g.}$ ) of synthetic coenzyme, 704.

#### *The Activation of Apoenzyme by Natural, Synthetic, and Pyridoxal Codecarboxylase*

This approach resembles that of Epps (3), i.e. the enzyme is isolated in a cell-free state in the active form, and the coenzyme is removed and replaced by codecarboxylase preparations. If the codecarboxylases

obtained by the three methods were not identical, one might reasonably expect that the synthetic coenzyme and the pyridoxal coenzyme would not replace the natural codecarboxylase in the enzyme system from which the latter had been removed.

To test this possibility two types of cell-free apoenzymes were prepared from the active cells. One of these still contained the system capable of converting pyridoxal plus ATP into coenzyme whereas the other was activated only by the preformed coenzyme. The exact mechanism involved in the resolution of the enzyme-coenzyme complex is not clear, but sufficient resolution for studies of the coenzyme preparations can be obtained by aging the cell-free enzyme preparations followed by dialysis. The dissociation constant of the codecarboxylase is so low that preparations can be carried through several steps in the purification before the slightest evidence of dissociation is noted. Dialysis is of no value in the early stages of purification but is useful with aged preparations. The greater part of the resolution appears to be due to a gradual breakdown of the coenzyme when the cell-free enzyme preparations are held in the refrigerator. Epps (3) also used an "aging process" for the complete "dissociation" of the enzyme-coenzyme complex. To date the most successful method of "dissociating" the codecarboxylase from the enzyme has been to adjust the cell-free extract to pH 5.0 and hold in the refrigerator for 10 to 15 days, then to dialyze for 10 to 15 hours against pH 5 buffer at 28°C. Although this procedure is usually successful, some preparations have retained most of their activity for as long as a month. When the aging process is used, the decrease in the activity of the enzyme preparation compares roughly with the rate of codecarboxylase decomposition as indicated in Table IV. Full activity of the enzyme can be restored by the addition of codecarboxylase.

As stated previously two types of apoenzymes have been prepared from the cell-free extract obtained from active cells (Fig. 1, Stage A). Figure 4 illustrates the type of data obtained with the two preparations. In the first of these (Fig. 4A) the enzymes capable of converting pyridoxal plus ATP into coenzyme are still present. As the figure shows, pyridoxal alone has no effect upon this system but the natural codecarboxylase and pyridoxal plus ATP are highly active. In the second preparation, Fig. 4B, the enzymes which convert pyridoxal plus ATP into codecarboxylase have been largely destroyed. Therefore, only the natural and the synthetic codecarboxylase preparations are

active. The separation of the enzymes which convert pyridoxal plus ATP into codecarboxylase from the tyrosine decarboxylase was accomplished by diluting the cell-free enzyme obtained from acetone-dried cells and allowing it to age in the refrigerator for 25 days. Like

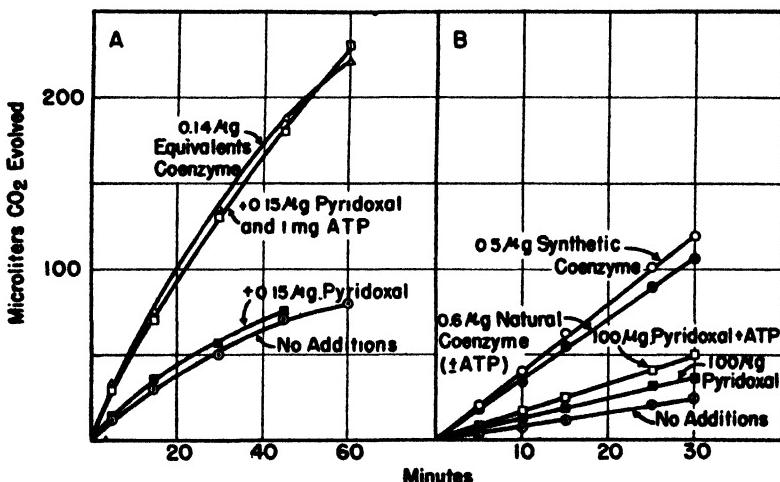


FIG. 4

Activity of Codecarboxylase Preparations on Apoenzyme from Cells Grown on Complex Medium

Per Warburg flask: Side arm, 0.5 ml. *M*/30 tyrosine tipped at zero time.

Main compartment: 0.5 ml. 0.2 *M* acetate buffer pH 5.5; enzyme as A or B; water to 3 ml. 28°C.

Part A. Apoenzyme from vacuum-dried cells: 8.05 mg. protein (Stage A, Fig. 1) extracted from ground cells with 12.5% KCl. Held at 0°C. for 14 days, then dialyzed 20 hours at 28°C.

Part B. Apoenzyme from acetone-dried cells: 2.01 mg. protein (Stage A, Fig. 1). Extracted by autolysis procedure, aged at 0 to 5°C. for 14 days; dialyzed 20 hours at 28°C.

the procedure for preparing the apoenzyme, this is not always successful.

From these data it is concluded that the cell-free tyrosine decarboxylase obtained from cells grown in a complex medium is comparable to the system described by Epps (3) and Gale and Epps (1). The data also indicate that the natural and synthetic codecarboxylase preparations—and in those cases where the necessary auxiliary

enzymes are present, the pyridoxal codecarboxylase—are all active and not distinguishable in their coenzyme activity for tyrosine decarboxylase. It is therefore suggested that the codecarboxylase of Gale and Epps (1) is a pyridoxine derivative.

### CONCLUSIONS

The preparation of tyrosine decarboxylase from *Streptococcus faecalis*, strain R, in a cell-free state and the resolution of this enzyme into apoenzyme and coenzyme is described.

The coenzyme is identified as a derivative of pyridoxal, and a procedure is given for the chemical conversion of pyridoxal into the coenzyme in approximately 5% yield.

It is concluded that the naturally occurring coenzyme, described by Gale and Epps (1), is a pyridoxin derivative because it can be replaced in a variety of enzyme systems by coenzymes derived from pyridoxal and because on hydrolysis it yields a material which behaves with the tyrosine decarboxylase enzyme as does pyridoxal.

An assay system for codecarboxylase or for pyridoxal is described. The method employs tyrosine decarboxylase apoenzyme obtained from cells of *Streptococcus faecalis*, strain R, grown in a medium deficient in the vitamin B<sub>6</sub> group.

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# Lipids in the Central Nervous System of the Honey Bee

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Received March 1, 1945

## INTRODUCTION

Until quite recently entomologists in general believed that insect nerves lacked myelin sheaths. Using histochemical and optical methods, Richards (1943, 1944) reported the presence of extremely thin "lipoprotein" nerve sheaths in insects. These show optical properties similar to those of the nerve sheaths of crustacea, annelids, and molluses, and similar to those of "non-myelinated" fibers of vertebrates. The myelinated and non-myelinated fibers of vertebrates differ from one another in size and in relative amounts of lipid and protein in their sheaths. Accordingly, it has been suggested that all nerves of all animals have sheaths which are of essentially the same structure (Schmitt and Bear, 1939).

Lipids have been extracted and identified from vertebrate nerves; it has sometimes been assumed that the same lipids are present in invertebrate nerves although no analyses have been made. The present paper deals with the results of determinations of the lipids of bee "brains." The small amount of available material made it necessary to use microanalytical methods to, and in one case beyond, their reported limits of accuracy. Duplicate determinations on a given sample could rarely be made. The figures must, therefore, be considered as no more than a first approximation; their individual validities will be indicated. They do show, however, that the same groups of lipids can be extracted from bee "brains" as from vertebrate brains, and that the relative concentrations of extractable lipids, including sterols, fatty acids, and phospholipids, are not greatly different in bee "brains" from those in vertebrate brains prior to, or at the time of, the appearance of visible medullation.

### EXPERIMENTAL METHODS

Approximately 1300 honey bees (*Apis mellifica*, young adult workers of early spring brood) were decapitated and the "brains" (supraoesophageal ganglia, circumoesophageal commissures and suboesophageal ganglia) dissected out in physiological salt solution.<sup>1</sup> Care was taken to remove the various head glands, the oesophagus and the pigmented regions of the eyes and ocelli. Finally, the investing sheath (neural lamella) was peeled off to assure clean ganglionic preparations. Eight to twelve dissections were allowed to accumulate in the salt solution. Then, within less than one and one half hours after removal from the bees, they were rinsed quickly in distilled water to remove excess saline, dried *in vacuo*, and sealed in evacuated glass ampoules.

The material was transferred from the ampoules into tared test tubes and weighed. In order to secure the maximum information from these small samples (41–71 mg.), it seemed advisable to try to obtain at least a partial separation of the lipids by differential solubility. As shown in the scheme of analysis (Fig. 1), the solvents used were, in order: acetone, ether, and a mixture of three parts of methyl alcohol to one part of chloroform. All solvents used were reagent grade chemicals. During the extraction the tubes were kept at 5°C. Successive 1 ml. portions of a given solvent were added to the dry tissue. Each portion remained in contact with the tissue for 24 hours or more and was then poured off. When successive weights of the residue (dried over P<sub>2</sub>O<sub>5</sub>) showed no change, the extraction with a given solvent was considered to be complete. Microscopic examination indicated a reasonably satisfactory separation by acetone. The acetone extract, on evaporation, yielded birefringent crystals typical of sterols (m. p. > 100°C.), other crystals typical of neutral fats or fatty acids (m. p. 70–80°C.), and clear oil droplets. There were a few small round, seemingly amorphous, masses giving a polarization cross. These occurred particularly in sample No. 3, and were probably phospholipid. The ether extract showed no crystals but did show masses of amorphous substance, samples of which, on addition of water, gave myelin figures.

By this initial separation of the lipids it was hoped that errors in determining one constituent in the presence of all the others would be minimized. Procedures were combined so that the determination of several different constituents could be carried out on the same aliquot. In all cases where the separation and washing of a precipitate was necessary, centrifugation, rather than filtration, was used. Blanks on the reagents used, including the solvents, were carried through all procedures. All colorimetric measurements were made with a Beckman Spectrophotometer.<sup>2</sup>

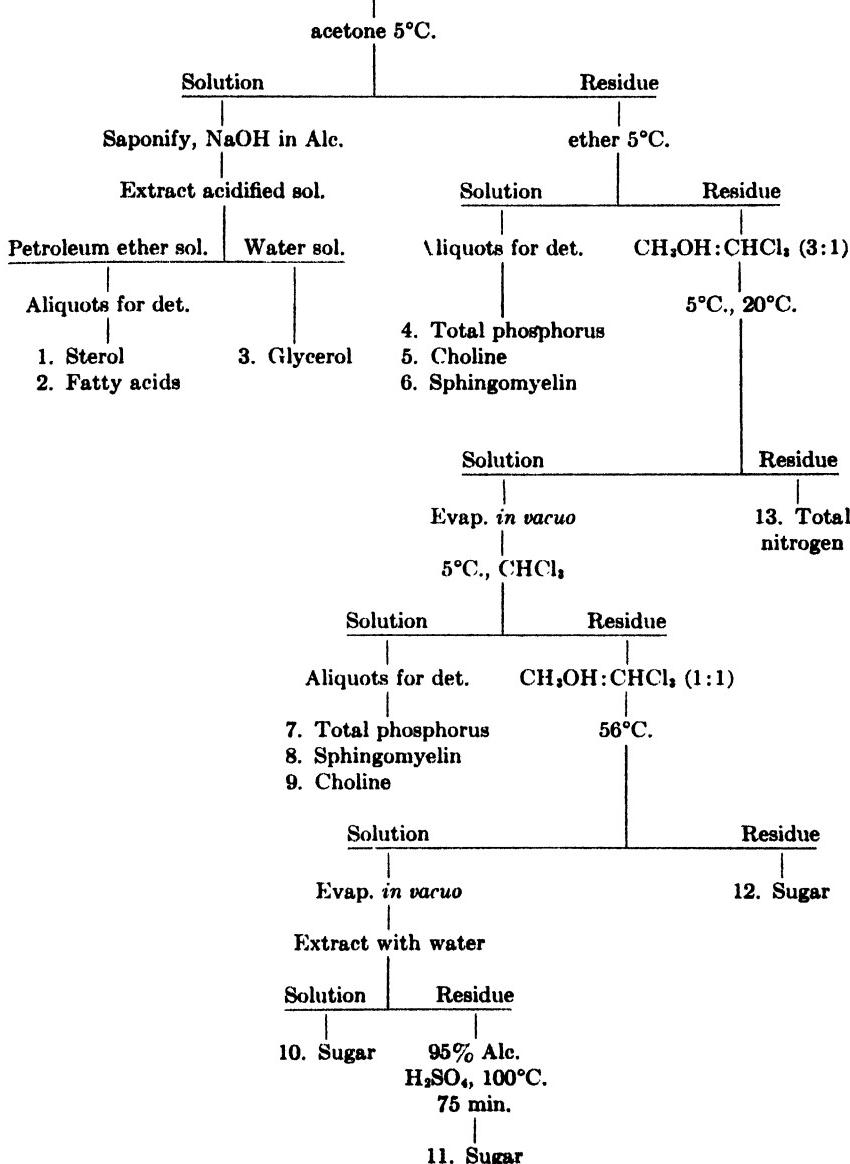
The material extracted with acetone was saponified and the glycerol estimated in the final aqueous solution by the methods described by Voris, Ellis, and Maynard (1940). The acidified solution was extracted with petroleum ether, and aliquots of the petroleum ether were used for the determination of the fatty acids by Bloor's

<sup>1</sup> The majority of these dissections were made for us by Miss Jane L. Weygandt.

<sup>2</sup> This instrument was obtained through a grant from the Carnegie Corporation Fund used for the Coordination of the Sciences at Bryn Mawr College.

## SCHEME OF ANALYSIS

## Dehydrated Bee Brains



(1928) oxidative method, and for the determination of sterols by the Liebermann-Buchard reaction as modified by Ireland (1941).

The ether and chloroform-methyl alcohol solutions were evaporated *in vacuo* and the residue taken up in chloroform. Aliquots of the chloroform solutions were used for the determinations of phosphorus, choline and sphingomyelin. The method of Fiske and Subbarow, as modified by King (1932), was used for phosphorus. The color developed was compared with standards at 720 m $\mu$  (Teorell, 1931). Choline was determined by Beattie's ammonium reineckate method, as modified by Thornton and Broome (1942) after hydrolysis of the phospholipids in methanol saturated with dry HCl gas (Thannhauser, Benotti and Reinstein, 1939). Sphingomyelin was estimated by the method of Thannhauser, *et al.*, 1939. The sphingomyelin-reineckate precipitates were washed three times with 1 ml. portions of cold methanol followed by three washings with a cold mixture of equal parts of chloroform and methanol. This procedure was based upon the finding of Artom and Fishman (1943) that chloroform removed free phospholipid. Chloroform alone could not be used because of the difficulties encountered in centrifuging. Phosphorus was determined in the sphingomyelin-reineckate precipitate.

In order to estimate the concentration of cerebrosides, sugar determinations were carried out on the chloroform-methanol extracts by Bruckner's (1941) orcinol-H<sub>2</sub>SO<sub>4</sub> reaction. These determinations were performed separately on various fractions within the extract, as shown in the Scheme of Analysis.

Nitrogen determinations were carried out on the residue of the dry brains after extraction of the lipids was completed. A micro-Kjeldahl method was used. The digestion mixture and the time of digestion were those recommended by Chibnall, Rees, and Williams (1943).

## RESULTS

The results are summarized in Tables I and II. Table I lists the amounts extracted by each solvent and the times of extraction. Table II shows the results of the analyses carried out on the various aliquots. The percentages of extracted material in Table II are based on the total weight extracted as shown in Table I.

The small amount of material available made it difficult to carry out determinations of all the constituents on each sample. Many of the values reported (see footnotes to Table II) are partial; that is, the determinations were carried out on only one extract when the constituent in question might also have been present in other extracts.

The actual amounts of each constituent determined were within the range reported for the method used with the exception of glycerol. The amounts of glycerol determined in the present work ranged from 0.05 to 0.14 mg. Glycerol standards in that range gave from 85 to 95% yields. Nevertheless, in dealing with such small amounts the errors may be large. "Neutral fat" is calculated as glycerol  $\times$  9.6.

The sterol values seem more reliable. The aliquots used yielded 0.08 to 0.3 mg. of sterol, measured against cholesterol standards. Preliminary hydrolysis did not change the apparent sterol concentration. Bloor (1943) states that variant forms of sterols are present in lower animals and in insects. Measurement against a cholesterol standard may, therefore, introduce some inaccuracy.

Fatty acid values (0.5 to 0.8 mg.) in individual determinations fell in the range of the Bloor (1928) oxidative method. Standard determinations of this magnitude gave 96 to 100% yields. The "fatty acids"

TABLE I  
*Lipids Extracted by Successive Solvents*

Sample number	1 ca. 200	2 ca. 200	3 ca. 200	4 305	5 ca. 300
Number of brains	41.2	50.6	45.8	71.4	68.5
Total dry weight in mg.					
Acetone soluble fraction					
Time of extraction in days	2	4	50	17	17
Weight in mg.	2.6	4.0	6.4	3.4	3.6
% of dry weight	6.3	7.9	14.	4.8	5.3
Ether soluble fraction					
Time of extraction in days	10	34	5	24	24
Weight in mg.	5.7	5.8	0.9	11.1	9.5
% of dry weight	13.8	11.5	2.	15.6	13.9
CHCl <sub>3</sub> : CH <sub>3</sub> OH fraction					
Time of extraction in days	2*	6†	27	35	28
Weight in mg.	7.8	8.3	10.6	16.4	13.5
% of dry weight	18.9	16.4	23.1	23.0	19.7
Total lipid extracted in mg.	16.1	18.1	17.9	30.9	26.6
% of dry weight	39.1	35.7	39.0	43.3	38.9

\* Followed by three 20 minute extractions at 60°C.

† Followed by six 20 minute extractions at 60°C.

may include free fatty acids, fatty acids from neutral fat, and possibly a small amount from the phospholipids.

The phosphorus determinations were well within the reported limits of the method. The factor, 25, was used for the conversion of phosphorus values to phospholipids. The phosphorus in the sphingomyelin-reineckate precipitate was converted to sphingomyelin by the use of the factor 26.8.

The choline values are to be considered minimal. This is because these determinations were performed only on the ether extract and because corrections were not made for possible losses in pumping off

TABLE II  
*Composition of Bee "Brains"*

Sample number	1	2	3	4	5
<b>Sterols (as cholesterol)</b>					
total in mg.	0.53 <sup>a</sup>	0.81 <sup>a</sup>	0.81	0.52	0.90
% of extracted material	3.3	4.5	4.5	2.9	2.9
% of dry weight	1.3	1.6	1.6	1.1	1.3
<b>Neutral fat</b>					
total in mg.		2.2	1.6	0.72	0.44 <sup>b</sup>
% of extracted material		12.	8.9	2.3	1.7
% of dry weight		4.3	3.5	1.0	0.64
<b>Fatty acids<sup>c</sup></b>					
total in mg.		2.2	3.0	1.8	1.5 <sup>b</sup>
% of extracted material		12.	17.	5.8	5.6
% of dry weight		4.3	6.5	2.5	2.2
<b>Phospholipids</b>					
total in mg.	2.8 <sup>d</sup>	1.4 <sup>d</sup>	1.4 <sup>d</sup>	7.2	12.7 <sup>e</sup>
% of extracted material				40.	41.
% of dry weight				16.	18.
<b>Sphingomyelins</b>					
total in mg.				0.45 <sup>d</sup>	1.05
% of extracted material				2.5	3.4
% of phospholipid				6.2	8.3
% of dry weight				1.0	1.5
<b>Choline phospholipid</b>					
total in mg.	0.62 <sup>f</sup>		1.98 <sup>f</sup>		
% of extracted material	3.9		10.9		
% of dry weight	1.5		3.9		
<b>Cerebrosides</b>					
<b>Nitrogen</b>					
% of dry residue	15.3		14.9	14.3	15.3
				0	0
					0

<sup>a</sup> not hydrolyzed.<sup>b</sup> values slightly low due to loss of some acetone extract.<sup>c</sup> includes fatty acids from neutral fat.<sup>d</sup> in CH<sub>3</sub>OH : CHCl<sub>3</sub> extract only.<sup>e</sup> does not include possible phospholipid in acetone extract.<sup>f</sup> in ether extract only.

the methanol and in washing the choline-reineckate precipitate. Choline phospholipid is calculated as choline  $\times$  6.65.

We found no evidence for the presence of cerebrosides in the bee "brains." Appreciable amounts of sugars were found only in the aqueous extract of the chloroform-methanol extract (Scheme of Analysis, No. 10). The extract, treated with orcinol-H<sub>2</sub>SO<sub>4</sub>, showed an absorption curve due to the sugars present. This curve did not cor-

respond with that of d-galactose but did show the presence of pentose and hexose sugars (Sørensen and Haugaard, 1933).

### DISCUSSION

So far as we know these are the first analyses published for any invertebrate nervous system. The available literature is concerned with the central nervous system of vertebrates which, except for embryonic stages, is heavily myelinated. Some representative sets of data are assembled in Table III. Inspection of these figures shows that the central nervous system of bees contains the same types and roughly the same proportions of lipids as are found in vertebrate brains except for cerebrosides. The sterol value is low. Comparison of our data from bee brains with the results from analyses of nervous tissue from vertebrate embryos reviewed by Needham (1931) and with the data given in Table III suggests that the bee "brain" is remarkably like the brain of a young vertebrate prior to the appearance of visible medullation. The results seem to affirm the statement of Hilditch (1940), "on the whole it seems likely that insects . . . lay down fats somewhat similar in type to those produced by mammals."

It would be interesting to compare the extractions from bee "brains" with extracts from non-myelinated fibers of adult vertebrates. Optical analyses (Schmitt and Bear, 1939; Richards, 1944) suggest a close similarity between these two, but no chemical analyses have been reported on the unmyelinated nerves or ganglia of adult vertebrates.

Our extractions were necessarily made from whole "brains." As such they contain equivalents to both the grey and white matter of vertebrate brains and spinal cords. The white matter of vertebrate brains, *i.e.* the fiber tract regions, contains a much higher percentage of lipids than the grey matter due to the high lipid content of the nerve sheaths. Comparison of our extraction data with the optical properties of living insect nerves (Richards, 1944) suggests that a large part of the lipids are located in the sheaths of the bee's nerves but presumably an unknown fraction would be obtained from the nerve cells and fibers themselves. It seems likely that most or possibly all of the nerve lipids of insects are "bound" to proteins—histochemical and optical methods have revealed no "free" lipids (Wigglesworth, 1942; Richards, 1943, 1944).

A considerable range of variation is shown by the data from differ-

TABLE III  
*Comparative Data on the Brains of Various Animals*  
 All figures given as percentages of the total dry weight

Animal	Total lipid	Acetone extract			Ether and $\text{CH}_3\text{OH}:\text{CHCl}_3$ extracts					Total Protein	Authority
		Total	Sterol	Neutral fat	Total phospholipid	Sphingomyelin	Lecithin	Cephalin	Cerebroside		
Human											
3 mo. fetus	22		7.2		12.9					0	46.6
7 mo. fetus	26.		10.3		13.1					0	42.1
1 mo. child	31.		12.9		16.3					0	38.3
3 mo. child	33.		13.1		13.4					2.3	40.8
8 mo. child	36		6.4		22.3					3.4	42.9
21 yr. adult	55.		16.0		25.0					5.7	35.4
33 yr. adult	55.		22.5		24.6					5.6	35.4
35 yr. adult	56.		15.0		25.3					9.5	33.1
67 yr. adult	56		15.0		27.2					8.0	35.0
Human cerebrum	54.4		10.0		28.3					6.63	37.7
cerebellum	50.9		6.4		25.0					7.43	40.4
Pigeon											
cerebrum	41.3		8.9		23.5						46.1
cerebellum	35.1		7.5		22.4						51.0
Frog summer											
winter											
experimental										0	
Dog—range	21.2		3.7		13.0					3.6	
	24.1		7.0		18.1					to 18	
Rat—range	22.8		4.8		12.6						
	21.9		7.2		20.4						
Human adult (1)					35.5	4.51	4.94	26.05			
adult (2)					26.3	6.82	4.68	14.80			
Dog					20.0	4.	12.	4			
Human											
7 mo. fetus	23.9	6.8			16.9	0				0.02	
new born	20.7	4.4			15.8	0				0.05	
13 mo. child	34.7	8.0			24.8	1.9				1.5	
14 mo. child	28.9	5.6			20.2	1.1				2.75	
old man	46.0	13.4			25.9	0.9				6.4	
Human spinal cord	14.8				32.1	2.6				5.1	
	19.7				21.9	2.8				6.2	
Rat average			8.95		23.6						
Beef (1)	49.4	13.6	10.8	2.84	24.3	5.05	6.65	12.57	11.55		
(2)	53.8	12.8	9.7	3.10	28.5	4.88	7.46	16.14	12.47		
Honey bee average	39	7.4	1.3	2.5	17.	1.5	2.	ca 12	0	56.	
range	35.7	4.8	0.8	0.6	—	1.0	—	—	0	54.	
	43.3	14.0	1.6	4.4		2.0	—	—	0	60.	Present paper

ent aliquots (Table II). How much of this variation is due to technical errors and how much to differences between the aliquots is not known. Even when macromethods are used considerable variation is shown

by the data from vertebrates—even the data within a single paper (Table III).

An average of 39% of the dry weight was extracted by the three sets of solvents. Approximately two-thirds of this total was identified further, leaving approximately one-third as unknown. Most of the material in the acetone and ether extracts was accounted for but less than half of the large  $\text{CH}_3\text{OH} : \text{CHCl}_3$  extract was identified. Schuwirth (1940) and others have likewise had considerable percentages of unknown material in  $\text{CH}_3\text{OH} : \text{CHCl}_3$  extracts from human brain and spinal cord.

Although the total phospholipid values seem reasonably accurate, the group was not satisfactorily fractionated. Approximations are presented in Table III. We can point out that the lecithin and sphingomyelin figures seem to represent only about one-fourth of the total phospholipid, and so presumably the cephalin value will be relatively high as it is in vertebrates.

The values for neutral fat are the most questionable of all the determinations presented. It does seem, however, that some neutral fat is present although no free droplets can be found by histochemical methods (Wigglesworth, 1942; Richards, 1943).

Fatty acids are, of course, to be expected after saponification. Whether any of these are present in the living "brains" in a free state is not known. Certainly most of the fatty acid values come from the hydrolysis of the neutral fat and the small amount of phospholipid present in the acetone extract.

Protein estimations were made primarily to check on the completeness of lipid extraction. Judging from the nitrogen values, the lipid extraction must have been essentially complete, and almost all the residue must have been protein.

#### SUMMARY

Lipids were extracted and analyzed from the head portion of the central nervous system of approximately 1300 honey bees. Standard micromethods were used to determine sterols, glycerol, fatty acids, phosphorus, choline, sphingomyelin, and sugars. Nitrogen determinations were carried out on the residue. Values for neutral fat, total phospholipids and proteins were calculated. No evidence was found for the presence of cerebrosides.

The data are compared with data from various vertebrates. It is concluded that the lipids of the insect nervous system are, in general, roughly comparable to those from the brains of young vertebrates prior to the appearance of visible medullation.

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# Microdetermination of Dicarbonyl Compounds On the Coloring of Nitro Compounds in Alkaline Solutions \*

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Received March 6, 1945

## INTRODUCTION, HISTORICAL, THEORETICAL

Compounds of 2,4-dinitrophenylhydrazine (DN),† easily obtained by the method of Curtius and Dedichen (1), have been prepared with simple aldehydes and ketones under conditions which cannot be attained in biochemical analysis (1, 2, 3). The application of DN to metabolism problems, particularly as a reagent for pyruvic acid and its homologues, has been demonstrated by Neuberg and his associates (4).

The extremely slight solubility of 2,4-dinitrophenylhydrazone (DNH) of keto acids, and the ease with which these compounds may be separated from their analogues with other carbonyl compounds, permits gravimetric determination of keto acids in amounts as low as 2.5 mg. (4, 5, 6). Although there are advantages to be gained by isolating these derivatives and identifying individual substances by their melting points, which can now be carried out with quantities in  $\gamma$ -range (7), the color reactions indicated by Neuberg and Kobel (4) are usually employed as routine colorimetric determinations. This procedure for detecting keto acids involves solution of their DNH in  $\text{Na}_2\text{CO}_3$ , precipitation of the keto acid by mineral acid, extraction with ethyl acetate or other solvents and renewed formation of their brownish red sodium salts.

\* Presented at the Cleveland Meeting of the American Chemical Society, April 6, 1944.

† In this paper the following abbreviations are used:

- |     |                                  |
|-----|----------------------------------|
| DN  | = 2,4-dinitrophenylhydrazine     |
| DNH | = 2,4-dinitrophenylhydrazone     |
| PN  | = <i>p</i> -nitrophenylhydrazine |
| PNH | = <i>p</i> -nitrophenylhydrazone |

Wherever it is possible to derive the osazone from osones or analogous 1,2-dicarbonyl compounds without straining, they will be called bis-hydrzones.

Since the introduction of this procedure, numerous modifications and submodifications have been described, none of which has introduced any essential change: e.g., those by Case (1932), Peters and Thompson (1937), Penrose (1937), Jewett and coworkers (1937), Lu (1939), Kato (1941), Sealock (1941), Long (1942), Friedeman (1943), etc. The procedures described by Pi-Sufier and Ferrán and by Bueding and Wortis (8) are easy to use and well adapted to prevalent conditions.

The blue to violet color reaction of *p*-nitrophenylosazones (bis-*p*-nitrophenylhydrazones) when dissolved in alcoholic alkali is also known. This reaction for a simple *p*-nitrophenylhydrazone was described by Gnehm and Benda (9) in 1896 and again, without crediting either this or other publications, in 1899 by Bamberger and Hyde.

As has been previously stated, this reaction occurs with the PNH of glyoxal, methylglyoxal, and dimethylglyoxal. The bis-DNH of these simple substances give a deep violet color reaction. This reaction, described by Neuberg and Kobel (4), has been evaluated microchemically for the determination of methylglyoxal by Barrensheen and Dreguss (10). It is astonishing that this reaction has not been generally applied, as it can be used for the colorimetric determination of all substances forming bis-DN (or bis-PN) derivatives.

The simple dicarbonyl compounds of the type represented by glyoxal, methylglyoxal, or dimethylglyoxal, form bis-DNH quantitatively (4, 11), while the sugars, with rare exceptions, do not form phenyl- or nitrophenyl-osazones quantitatively. Only certain triose derivatives seem to give quantitative reactions. The quantitative separation of methylglyoxal-bis-DNH from fermentation mixtures, described in earlier papers (4), may be considered partly due to formation of the osazone from natural triosephosphoric acids. It has, in fact, been shown that methylglyoxalnitrosazones can be obtained quantitatively from trioses as well as from synthetic *dl*-glyceraldehyde-phosphoric acid (12). In like manner, if Weygand's observation (13) be noted, the osazones are more easily produced after addition of aromatic amines (*p*-toluidin or *p*-phenetidin) because of intermediate formation of substituted amino ketoses by the Amadori rearrangement, quantitative yields cannot be obtained. However, this is the case with osones, for E. Fischer (14) found osones give good yields of phenylosazones, the reaction becoming quantitative when DN is used instead of the simple hydrazines. This could be verified in the case of *l*-arabinosone, *d*-xylosone, *l*-sorbosone, *d*-galactosone, maltosone, and lactosone, results on which will appear in a forthcoming paper.

The dinitrophenylosazones of the sugars corresponding to these osones are soluble in sodium ethylate with a deep blue or bright violet color. The same reaction is also given by the DNH of higher 1,2-diketones, as demonstrated in the case of the acetylcaproyl and acetylbenzoyl (see p. 225) compounds.

Definitely different from this are the reactions shown by the unknown oxidation products of malic acid (Pucher, *et al.*, 15) and by dehydroascorbic acid (Penney and Zilva, 16). Here, advantage is taken of the solubility of the DNH of a carboxyl compound in aqueous NaOH. These solutions show a reddish or blue color, analogous to that of the DNH of pyruvic acid and its homologues. Roe and coworkers (17) have developed a red color by addition of strong sulfuric acid to a mixture of dehydroascorbic acid and DN. It is not clear whether this color test is due to the real dinitrosoazone or the hydrazone of ascorbic acid, described by Kotake and Nishigaki (18) and by Herbert, *et al.* (19).

With increase of our knowledge of carbohydrate metabolism, the importance of acetyl methylcarbinol and 2,3-butylene glycol formed by its reduction, may be considered similar to that given for pyruvic acid and its homologues more than thirty years ago.

The formation of acetoin and of acyloins in general, as well as 2,3-butylene glycol, has been explained from a study of processes occurring in yeast cells (20, 21). The relation of the acyloins, both to acetaldehyde and pyruvic acid and to other  $\alpha$ -keto acids can then be stated (21). These compounds have been found as metabolism products of numerous microorganisms of green plants and, since 1930, of animal organs (Lemoigne, Gorr, Tomiyasu, Peters, Green, Greenberg, Berg, Stotz, and Westerfield, *et al.*). In principle, the mechanism of formation is always the same, and therefore, the micro-analytical determination of these products of the "butylene fermentations" is of interest.<sup>1</sup>

Acetoin can be transformed quantitatively to diacetyl by oxidation with  $\text{FeCl}_3$ . The same effect can be produced with 2,3-butylene glycol—recently of industrial interest—by simultaneous addition of  $\text{Br}_2$ . The method, originated by Lemoigne, was later developed by Kluyver, Donker, and Visser t'Hooft (24) and van Niel (25). This procedure permits separate quantitative determinations of acetoin, 2,3-butylene glycol and diacetyl. The modus procedendi has been adapted to micro methods by different authors.

In the course of investigations on fermentations carried on at Dahlem, methods have been developed which permit the isolation, identification, and determination of the butylene products balance. These methods have made extensive use of bis-PNH and bis-DNH reactions. The microchemical determination of these substances, described in this paper, is based on their transformation into diacetyl-

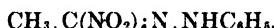
<sup>1</sup> As is well known, diacetyl and acetoin, respectively, are responsible for the aroma of butter, bread (22), and tobacco (23).

bis-DNH and depends on the deep violet color of their solutions in sodium ethylate for their colorimetric evaluation.

This latter procedure<sup>2</sup> is applicable to many dicarbonyl compounds. The reason for this remarkable color phenomenon is not completely understood, but in most cases colored salts are produced (see pp. 217, 218).

P. Pfeiffer has expressed the opinion that introduction of nitro groups weakens the color of a compound (hypsochromic effect), but in dye chemistry especially, it is believed that bis- and poly-nitro compounds are more deeply colored than mono-nitro compounds. However, generally this is not so. E.g., 4-nitrophenylosazones of the sugars are almost all deep-red to brick-red while the corresponding 2,4-dinitrophenylosazones are yellowish-brown or orange, and not deep red. Similar differences can be observed with the alkali and, to an even greater extent, with the alcoholic alkali solutions of these compounds as well as those of some mono-hydrzones.

The literature concerning this field, important for the micro-analyst, is incomplete and incorrect. V. Meyer, the discoverer of the aliphatic nitro compounds, first described the colorations occurring on contact of nitro compound hydrazones with alkali in 1875. The phenylhydrazone of nitro-acetaldehyde,



is golden yellow; its solution in alkali is blood-red (26). Analogy exists, according to V. Meyer (27) for reaction of the phenylhydrazone of nitropropionaldehyde,



with alkali. The 3-nitrophenylhydrazone of nitroacetaldehyde,



is yellow, but its alkaline solution is red (28). The *p*-sulfophenylhydrazones of nitroacetaldehyde and its homologues are yellow (Kappeler, 29); their alkaline solutions are deep red. The yellow DNH of acetone is, according to E. Fischer and Ach (30), as well as Curtius and Dedichen (1), soluble in alkali with a dark red color, the same being true in the case of the benzaldehyde compound (1). The phenylhydrazone of the methyl ester of 2,4-dinitrophenylglyoxylic acid,



is orange-red; its solution in alcoholic alkali is deep blue (31). Observations were

<sup>2</sup> The procedure differs from that used in determining the keto acids. For the determination of the latter, the solubility of the keto acid hydrazones in a  $\text{Na}_2\text{CO}_3$  solution is employed. In this solvent the osazones are insoluble as they are not derivatives of dicarbonyl acids. Therefore, *in principio*, they may be separated from the hydrazones. The osazones are, however, soluble in a Na-ethylate solution.

then made on acylhydrazides. The 2-nitrophenylhydrazide of formic acid,



is light yellow (Bischler, 32) and its alkaline solution is bluish-violet; the alkaline solution of the yellow 2,4-dinitrophenylhydrazide of acetic acid is deep red (1). As Pope and Hird (33) have shown, a carbamic acid derivate, the 2-nitro-4-methyl-phenylsemicarbazide,



which is sulphur yellow, produces a deep violet color with alcoholic KOH. Another type is presented by *p*-dinitrotartrazine. This substance, the bis-PNH of diketo-tartaric acid,



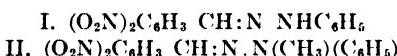
is soluble in dilute KOH with a deep indigo-blue color, as shown by Gnehm and Benda (9) in 1898.

Appearance of the nitro group in an aliphatic or aromatic radical seems to be unimportant for deepening of color in alkaline solution, as nitrophenylhydrazone of N-free carbonyl compounds and phenylhydrazone of nitro compounds both produce red, blue or violet alkaline solutions. No change other than the shade of color occurs, if both components are nitrated or if the phenylhydrazone is sulfonated. Colors are also produced when the compounds in question have neither a hydrazone nor osazone structure, *i.e.*, they may be nitrated phenylhydrazides, *e.g.*, the nitrophenylhydrazides of formic or carbamic acids, alkaline solutions of which are a deep violet.

Twenty four years after V. Meyer's and 3 years after Gnehm and Benda's publications, Bamberger and his coworker Hyde (34) described colors of mono- and bis-PNH in alkaline solutions. They verified the older findings and amplified them with observations on new substances. Thus, Hyde noticed that the *p*-nitrophenyl-osazones of the sugars behave similarly toward alkali as the bis-*p*-nitrophenylhydrazone of diketotartaric acid which is, in fact, an osazone (*p*-nitro-tartrazine). Wohl and Neuberg (35) stated that the simplest representative of these compounds, glycolaldehyde-*p*-nitrophenylosazone (= glyoxal-bis-PNH), is soluble even in strong aqueous NaOH with blue color. The same phenomenon is observed with alkaline solutions of rhamnosone-bis-*p*-nitrophenylhydrazone (36). The numerous (37) bis-PNH compounds prepared from methylglyoxal and its homologues, from galacto-arabinosone and from diacetyl are also soluble in alcoholic alkali with a deep-blue color.

Color phenomena are also manifested by many nitro compounds which apparently belong to totally different classes of compounds, but which may be taken into consideration, for the purpose of obtaining a comprehensive sifting clarification.

The phenylhydrazone of the methylester of nitroglyoxylic acid is yellow; its K-salt is cinnabar-red (38). 2,4-Dinitrobenzaldehyde-*p*-bromophenylhydrazone produces a deep blue color, and the corresponding derivative of 2,4,6-trinitrobenzaldehyde a green color in a NaOH solution (39). Violet and blue colors, respectively, are produced by the PNH of nitro-acetaldehyde (40) and the PNH of benzil (41), while a rose-red color is produced by the PNH of mesoxalic ester (42). The 2,4,6-trinitrophenylhydrazide of acetic acid is yellowish-green and is soluble in alkali with a deep red color (43); the solution of the 4-sulfophenylhydrazone of 2,4-dinitrobenzaldehyde in water is red, but on addition of alkali becomes green, blue, or violet, according to the strength of the alkali (44). These colors may be explained by salt-formations at the carboxyl and sulfo groups, but it also seems possible that there may be participation of the imido group. As an example of this last type, the reaction described by F. Sachs and his coworkers (39, 44) for the phenylhydrazone (I) and the methylphenylhydrazone (II) of 2,4-dinitrobenzaldehyde may be noted: II, containing a substituted imino group, is never colored by KOH, but I, which can form a normal or chinoid salt (see also p. 218), is soluble in alcoholic KOH with a deep blue color:



The conclusion previously drawn (see p. 215) is discoverable from statements scattered in the literature, mostly obtainable only from the original publications. To facilitate their finding, the names of the authors have been appended to the respective nitro compound, when the quotation in question can be easily located in "Chemical Abstracts," the "Chemisches Zentralblatt" and "Beilstein."

2,4-Dinitrobenzaldehyde-*p*-bromophenylhydrazone, Sachs and Kantorowiz (blue); 2,6-dinitrophenylacetone-phenylhydrazone, Borsche and Rantscheff (deep blue); formyl-2-nitrophenylhydrazone, Bischler (blue-violet); anthraquinone-mono-*p*-nitrophenylhydrazone, Kaufler and Suchanek (blue); 2,4-dinitrophenylacetic acid-phenylhydrazide, Pfeiffer (violet); phenylglyoxylic acid-2-nitrophenylhydrazone, Gastaldi (red); phenylglyoxal-carboxylic acid-2-nitrophenylhydrazone, Ciusa (dark red, warm); 2-nitrobenzene-sulfonic acid-2-nitrophenylhydrazide, Claasz (blood red); benzophenone-3-nitrophenylhydrazone, Ciusa (green); phenylglyoxal- $\omega$ -3-nitrophenylhydrazone, Ciusa (red); formaldehyde-PNH, Zerner (red-violet); benzaldehyde-PNH, Ciusa (a potassium salt  $\text{C}_{12}\text{H}_{10}\text{O}_2\text{N}_3\text{K}$ , crystallizing in dark-violet needles); 2-nitrobenzaldehyde-PNH, Bamberger and Fodor (permanganate red); hydroxy-ethoxy-methyl-propiophenone-PNH, v. Auwers (blue-violet); 3,4,5-tri-acetoxybenzaldehyde-PNH, Rosenmund and Zetsche (violet); glyoxalcarboxylic acid-PNH, Berl and Fodor (blue); benzoic acid-nitro-*o*-tolylhydrazide, Poncio and Maciotta (wine red); acetic acid-2,4,6-trinitro-*m*-tolylhydrazide, Giua (red); N-acetyl-compound of the same, Giua (deep dark red); benzoic acid-2-nitro-*p*-tolylhydrazide, Poncio and Maciotta (blue); benzoic acid-3-nitro-*p*-tolylhydrazide, Poncio and Maciotta (brown); benzaldehyde-4,6-dinitro-2,5-dimethylphenylhydrazone, Giua (violet-red); anisaldehyde-4,6-dinitro-2,5-dimethylphenylhydrazone, Giua (carmine red); benzaldehyde-2,4,6-trinitro-*m*-tolylhydrazone (Giua) (brick red).

That the hydrazone group alone can produce the color-deepening in alkaline solutions becomes evident from the *nitrated hydrazobenzenes*: 4,4'-dinitrohydrazobenzene,

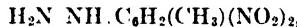


Werner and Stiasny (blue); 2,2'-dinitrohydrazobenzene, Green and Rowe (violet); 2,2'-dichloro-4,4'-dinitrohydrazobenzene, Green and Rowe (blue); 2,4,2'- and 2,4,4'-trinitrohydrazobenzene, Werner and Stiasny (blue); 2,4,2'4'-tetrannitrohydrazobenzene, Green and Rowe (blue); 2,4,6,4'-tetrannitrohydrazobenzene, Green and Rowe (blue); 2,4,6,2',4'-pentanitrohydrazobenzene, Green and Rowe (blue, and, depending on strength of the alkali, red and violet) Ciusa; 2,4,6,2',4',6'-hexanitrohydrazobenzene, Lehmann and Grandmougin (blue-violet); 4,6-dinitro-*m*-hydrazotoluylene, Giua (dark red); another derivative of hydrazobenzene, the N-methyl-N-phenyl-N'[2,4,6-trinitrophenyl compound]



Giua and Cherchi (grass-green).

It is very remarkable that, like the hydrazo compounds, even some hydrazines are soluble in alkali with totally changed colors: 4,6-dinitro-3-methylphenylhydrazine,



Giua (red); 2,4,6-trinitrophenylhydrazine, Lehmann and Grandmougin (deep red); 2,4,6-trinitro-3-methylphenylhydrazine,



Giua (brown); 4,6-dinitro-2,5-dimethylphenylhydrazine,



Giua (brick red).

This solubility in aqueous or alcoholic alkaline solutions accompanied by color changes of complicated nitrated hydrazines which were originally yellowish or orange becomes important in the analytical procedure (see page 220), as these effects must be considered when simple mono- and di-nitrophenylhydrazines are used as reagents.

The color changes which sometimes occur only on raising the temperature of the solutions, may be explained as a formation of different salts,<sup>3</sup> e.g. in the case of hexa-nitro-hydrazobenzene. From this compound, which is originally yellow, a green mono- and a bluish-violet dipotassium salt has been isolated (Lehmann and Grandmougin). The same fact can be stated for the penta-nitrohydrazobenzene (Lehmann and Grandmougin) and for the *o*- and *p*-nitrophenylhydrazones of phenylglyoxal-carboxylic acid (Ciusa),



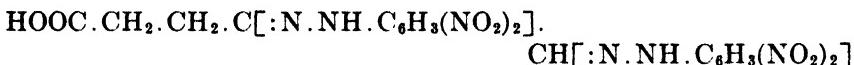
In their di-potassium salts,  $\text{C}_{15}\text{H}_9\text{O}_5\text{N}_3\text{K}_2$ , the imido group may also act as a salt-

<sup>3</sup> A similar explanation may be valid for the observations made 50 years ago by the first investigators (E. Fischer, Curtius, Feist) on the hydrazones of simple mono-carbonyl compounds, i.e., that their color in alkaline solution is considerably deepened with the quantity of NaOH added.

former, whereas, in the mono-potassium salts,  $C_{16}H_{10}O_4N_2K$ , only the carboxyl group acts thus, only the latter salt being formed with aqueous  $K_2CO_3$  solution. Vice versa, the di-potassium salts are changed into the mono-potassium salts by large amounts of water. A brick-red mono- and violet di-sodium salt is also obtained from cinnamoylformic acid-PNH,  $C_{16}H_{12}O_4N_2Na$  and  $C_{16}H_{11}O_4N_2Na_2$ , respectively (Ciusa and Bernardi).

We wish to refer to the decolorization of the blue or violet alkaline solutions of the bis-DNH and bis-PNH of dicarbonyl compounds by  $CO_2$  and the weakening of the color by dilution to extremely low concentration (see above and p. 219). These phenomena can be explained from the standpoint of dissociation of the salts of weak acids, *i.e.*, the dissociation can reach the point of decolorization, but the color may be regenerated by addition of large amounts of alcohol.

The carboxylated compounds, however, *e.g.*, the bis-DNH of methylglyoxalylacetic acid



are stronger acids and consequently the cornflower-blue or violet color, which is produced in aqueous alkaline or alkali carbonate solutions of these substances, does not disappear even when greatly diluted with water (Paul Mayer) (45).

Regarding aliphatic nitro compounds, one must take into consideration that, in addition to the (not well supported) formula of a carbazoxy ring,  $R \text{CH} \text{NOH}$ ,



there might be a transformation into azi-nitro-paraffins (isonitro-paraffins)



(Michael, 1888; Nef, 1894), the salts of which have been formulated by Kuhn and Albrecht a.o. (46) using the octet theory and electronic formulas.

However, the important statement that deeply colored chinoid tautomers are present in alkaline solutions, made by Hantzsch and Lister (47) and Hewitt, Johnson, and Pope (48), must be considered as applying to all nitro hydrazones, regardless of where the nitro groups appear. Consequently, the Na-salt of benzaldehyde-PNH may be formulated as follows:



and the Na-salt of *p*-nitrobenzaldehyde phenylhydrazone is expressed similarly. To all of which may be added effects produced by oscillation and especially by a resonance starting from the nitro group whose inductive function is most greatly accentuated in aromatic compounds.

It may be said, insofar as it is possible to derive a practical rule for the microchemical determination of dicarbonyl compounds, that 1,2-dicarbonyl compounds of biochemical analytical importance of the types



can be determined by the violet color produced by their bis-DNH in a sodium ethylate solution.<sup>4</sup>

The bis-DNH obtained in hydrochloric acid solution by precipitation with DN, is separated and washed with dilute HCl and water by centrifugation. The complete removal of excess DN eventually adherent is necessary (see p. 217 and p. 220) because of its interference with the color to be developed. When presence of keto-acid-DNH is suspected the bis-DNH must be washed on the centrifuge with weak  $\text{Na}_2\text{CO}_3$  solution until the solution is absolutely colorless. The purified bis-DNH is then dissolved in an alcoholic alkali solution in a volumetric flask, avoiding access of air.<sup>4</sup>

Principally, the procedure is the same for all dicarbonyl compounds; the colors have the shade of methyl violet and are similar to each other but not identical; they follow and approximate closely the Beer-Lambert law. The minimum limit of determination is 1-2  $\gamma/\text{ml}$ . of the dicarbonyl compound.

## EXPERIMENTAL

### *Reagents*

(1) DN, recrystallized from alcohol, M.P. 194-5°; 0.1 g. dissolved in 5 cc. of 2 *N* HCl. The solution is prepared on the boiling water bath and should be light yellow and absolutely clear after filtration. The hot solution is then diluted to 0.02% with 2 *N* HCl = 200  $\gamma/\text{ml}$ . This solution is again diluted 1:10 with *N* HCl before using, and now contains 20  $\gamma$ DN. The solution is stable at room temperature for about 8 days.

(2) 2 *N* HCl and *N*/10 HCl.

(3) A 0.3% solution of solid NaOH "pro analyse" in 96% alcohol or a Na-ethylate solution from 3 g. of metallic sodium in 1 liter absolute alcohol. (Dilutions of the alcoholic-alkaline color solution should be made with the sodium ethylate or with the 0.3% alcoholic alkali solution.) Each of the alkali solutions is fit for use only

<sup>4</sup> Small quantities of air do no damage, but larger ones provide a turbidity due to separation of  $\text{Na}_2\text{CO}_3$ . Decolorization follows introduction of  $\text{CO}_2$ , because the alkali carbonate is unable to produce the deeply colored Na salts even in the presence of alcohol.

as long as it is absolutely colorless and clear. They can be kept in the refrigerator for approximately one month. (A slight precipitate of  $\text{Na}_2\text{CO}_3$ , after settling, does not interfere with the use of the ethylate.)

### Procedure

From the simplest of diketones, diacetyl, 20  $\gamma/\text{ml}$ . still give a flocculent precipitate, which can be centrifuged off directly. Much smaller quantities (down to 0.8  $\gamma/\text{ml}$ .) can be determined by means of the following procedure. In a small test tube (4 in., Pyrex) 1 cc. of the test solution is mixed with 1-2 cc. of the dilute DN reagent and placed on the boiling water bath for one hour. After cooling, approximately 0.5 g. of kaolin is added, and the mixture, after thorough shaking, is vigorously centrifuged for 2-3 minutes. The absolutely clear supernatant solution is removed,<sup>5</sup> the precipitate mixed with N/10 HCl by stirring with a fine glass rod, rinsed, centrifuged, and finally washed similarly with water. After removal of the wash-water, the precipitate is thoroughly mixed with 3 ml. of the sodium ethylate solution, stirred well and allowed to stand for 5 minutes and then centrifuged. After removal of the first colored solution the precipitate is again eluted with 3 ml. of the sodium ethylate solution and centrifuged. Two extractions will usually suffice. The extracts are united and diluted to a convenient volume with the sodium ethylate solution; the colored solution is then compared with the standard solution. It is necessary to run a control parallel with the test to exclude the yellowish color originating from even the smallest traces of DN reagent and subtract its colorimetric value from the color value of the test. The blank is made with 1 ml. of distilled water plus 1-2 ml. of DN reagent, the subsequent treatment being exactly the same as that used in the test procedure. The washing with HCl should leave the kaolin sediment free from DN if possible. In this case, the extraction with alcoholic alkali will show no color. If there are traces of DN still present, a greyish-brown coloration appears, small gas bubbles develop, and the color quickly changes to a very faint but permanent yellow.

We have found that the application of kaolin as a precipitant, or perhaps an adsorbent, makes the determination of the dicarbonyl

<sup>5</sup> This supernatant liquid may serve to prove the presence of the necessary excess of the DN reagent. Addition of a few drops of dilute pyruvic acid or diacetyl solution must produce at least a distinct cloudiness.

compound possible even in cases of great dilution in which formation of the insoluble bis-DNH is not visible.

Some other substances, *e.g.*,  $\text{SiO}_2$ ,  $\text{TiO}_2$ ,  $\text{ZrO}_2$ ,  $\text{CaF}_2$ ,  $\text{CaSO}_4$ ,  $\text{BaSO}_4$ , bauxite, bentonite, powdered hematite, talcum, supercel, Fuller's earth, feldspar, give the same effect in acid medium, but they offer no advantage.<sup>6</sup>

From approximately equimolar solutions of glyoxal, methylglyoxal, diacetyl, and xylosone, containing respectively, 0.6, 0.75, 0.9 and 1.5  $\gamma/\text{ml.}$ , the bis-DNH compound is obtained in a form quantitatively separable only by means of the kaolin precipitation.

Semimicro gravimetric control determinations have shown that 5 mg. of the dicarbonyl compounds may be determined with a maximum loss of 2%;<sup>7</sup> *e.g.*, from 4.8 mg. of diacetyl we obtained 24.6 mg. of bis-DNH instead of 25.1 mg. as calculated. It is necessary to keep the mixture on the water bath for at least one hour. The statement given in the literature that DNH could react quantitatively and completely to form the bis-DNH products in a short time, is erroneous.

#### *Preparation of Standard Material*

The colors of the alcoholic-alkaline solutions of closely related dicarbonyl compounds are similar to each other but not identical (*cf.* pages 219 and 225).

The spectrum absorption measurements show the following results: The absorption maximum for the glyoxal-bis-DNH = 575, for methylglyoxal-bis-DNH = 561, and for diacetyl-bis-DNH = 551  $\text{m}\mu$ . Concentrations are given on p. 223. None of the solutions are dichroic.

We are indebted to Mrs. Dorothy Richmond of Interchemical Corporation, New York, for the determinations.

Standard solutions are required for colorimetric purposes. When 1,2-dicarbonyl compounds are difficultly obtainable, for preparing the

<sup>6</sup> In addition, the DNH can be precipitated from neutral solutions by  $\text{BaCO}_3$ ,  $\text{ZnCO}_3$ ,  $\text{Ca}_3(\text{PO}_4)_2$ ,  $\text{AlPO}_4$ ,  $\text{Fe}(\text{OH})_2$ ,  $\text{Pb}_3(\text{PO}_4)_2$ ,  $\text{PbC}_2\text{O}_4$ , etc., as well as by animal charcoal. Elution by sodium ethylate from the latter is, however, difficult, although it is relatively easy from the mineral compounds mentioned. Strain (49) has carried out chromatographic separations of the hydrazones of camphor and ionone from neutral solutions in petrol ether or benzene. Unfortunately, however, this procedure cannot be applied in the case of the slightly soluble osazones. In such cases solutions in pyridine, tetrahydrofurfuralcohol, etc. should be tested.

<sup>7</sup> Cf. the earlier statements by Neuberg, Kobel, and Simon (4, 6).

bis-DNH, the *monoximes*, so-called isonitroso- or oximido-ketones of the general formula



may be used as the starting material. In these monoximes (or in the correlated *dioximes*) the hydroxylamine radical is substituted quantitatively by the hydrazine radical on boiling with an excess of HCl-DN for two hours, the osazones being formed. The general osazone reaction is also applicable to the *acyloins*, various methods of formation being known. The osazone reaction is positive with these keto alcohols, and, as is known, has served in discovering the carbolicatic effect, *i.e.*, biochemical acyloin condensation. Originally, the reagent was PN, but changing to DN offers the advantage that its derivatives are in most cases still less soluble.<sup>8</sup> This also holds true for the osone bis-DNH, as represented by the corresponding sugar osazones.

Special directions are inserted here for preparing certain standards: *Glyoxal-bis-DNH*, from commercial glyoxal, according to Neuberg and Simon (11), or from a solution of glycolaldehyde, obtained readily by oxidation of ethylene glycol (H. Collatz and I. S. Neuberg) (12). The aldehyde is mixed with DN in HCl solution and the mixture heated for two hours on the water bath. The bis-DNH product is removed by suction, washed with dilute HCl and water and recrystallized from nitrobenzene, M.P. 326–328°C.

*Methylglyoxal-bis-DNH*: from methylglyoxal, which can be prepared quickly by the method of Neuberg and Hofmann (50). If the only purpose is the preparation of bis-DN, we advise use of the oxidation products of propylene glycol.<sup>9</sup> Recrystallized according to Neuberg and Kobel (4), the M.P. = 313°C.

*Diacetyl-bis-DNH*: from diacetyl or from its mono- or dioxime by heating on the water bath for at least two hours with a quantity of

<sup>8</sup> Neuberg and associates (4, 11).

<sup>9</sup> Dissolve 9 ml. of propylene glycol (1,2-propanediol) and 16 g. of K<sub>2</sub>CO<sub>3</sub> in 100 ml. of water, place in a cooling mixture and slowly add 5 ml. of Br<sub>2</sub> with constant shaking. Neutralize after 40–45 minutes, starting with finely powdered NaHSO<sub>3</sub> or Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and ending with a concentrated bisulfite solution. The liquid reduces Fehling solution strongly even when cold. It also contains acetol and lactic acid-aldehyde, both of which give the methylglyoxal derivative after heating two hours with an excess of DN in 2 N HCl.

DN in 2*N* HCl in excess of the theoretical. Such oximes are commercial products of satisfactory purity which are dissolved in 96% alcohol for use in the reaction. Recrystallized from nitrobenzene, M.P. 314–315°C.

These substances have also been prepared by other investigators (49, 51). The M.P.s recorded in the literature differ somewhat, as is common with such high melting compounds, particularly hydrazones. In addition, most of the DNH substances in the solid form exist as various polymorphic or syn-anti isomeric modifications.

For use in *colorimetric comparisons*, dissolve an exactly weighed amount of the standard, *e.g.*, 0.1 mg., in a 0.3% alcoholic NaOH or corresponding sodium ethylate solution, and dilute as necessary with the solvent. Substances were originally dissolved in pyridine,<sup>10</sup> an excellent solvent for all osazones (52) and causing no interference with development of the color. However, this procedure has proven unnecessary. Both control and test solutions should be kept at the same temperature, 18–25°C., as the shade of color is influenced somewhat by temperature.

The analytical work presented deals with determination of 1,2-dicarbonyl compounds as their bis-DNH. As previously mentioned, these yellowish-red substances are soluble in sodium ethylate with formation of a deep violet color. It is well known that the corresponding red bis-PNH are alkali-soluble with production of a deep-blue color. But this "rule" is only valid with reservations, *e.g.*, furil-bis-PNH and benzil-bis-PNH give a violet solution. Phenylglyoxal-bis-PNH and acetylbenzoyl-bis-PNH, having a "mixed" aliphatic and aromatic structure, exhibit an indigo blue color in alcoholic alkali solutions. Acetylcaproyl-bis-PNH, having approximately the same molecular weight as the corresponding acetylbenzoyl derivative, shows a strong indigo blue color. The color, therefore, is not a function of the mo-

<sup>10</sup> Pyridine forms non-characteristic brownish solutions. Potassium, sodium, strontium or barium ethylates, or strong organic bases act similarly to sodium ethylate. Solutions of glyoxal- or diacetyl-DNH in pure ethylene diamine, propylene diamine, 1,3-diaminobutane or benzyl triammonium hydroxide are indigo blue, the color changing to violet on addition of alcohol. Amino-alcohols, such as ethanolamine, 4-amino-2-butanol and 2-amino-1-butanol, combining, as they do, characteristics of both an alcohol and a base, produce the violet color directly. Although the action of these substances is of theoretical interest, compared with sodium ethylate, they offer no analytical advantage. There is ample chance for development of many modifications, even in the application of this method.

lecular weight. The acetylcaproyl-, acetylbenzoyl-, furil- and benzil-bis-DNH compounds are all soluble with a violet color.

In general, the introduction into the 4-nitro-phenylhydrazine of a second nitro group at the 2-position produces a distinct color change in the solution of the compound in question. Evidently DN can be considered as a PN substituted in meta position to the nitro- and in ortho position to the hydrazine radical. This raises the question of the color reaction of the parallel *o*- and *m*-nitrophenylhydrazones. The colors of these have been examined.

In investigating these phenomena another circumstance must be considered. It can be deduced from the observations of V. Meyer and coworkers (1875-1900), that the phenomenon of noteworthy coloration in alkaline solutions of nitrated hydrazones is not due to the presence of the nitrophenylhydrazine radical at all. The nitro group may be present in an entirely different part of the molecule, *e.g.*, the phenylhydrazones of nitroacetaldehyde and of 2,4-dinitrophenylglyoxylic acid methylester, the first substance being soluble in alkali with a deep red, and the latter with a deep blue color.

The facts serving as basic material for the foregoing problems are recorded in the following tables.<sup>11</sup> From these tables the regularity mentioned above (cf. p. 219) can be seen, the deepening of color being marked. In the case of 1,2-dicarbonyl compounds, it is not important whether the adjacent carbonyl groups appear at the end (glyoxals) or within the carbon chain (diketones). The diphenyl-1,2,3-triketo-bis-DNH, in which the carbonyl groups are vicinal, acts in the same manner, while acetonylacetone, a 1,4-diketone, acts differently. Monohydrazones assume the color producing property of the bis-hydrazone when the carbonyl component is nitrated. Hence, *p*-nitrobenzaldehyde-PNH and -DNH exhibit the same colors in alcoholic alkaline solutions as the glyoxal-bis-PNH and -DNH.

The following conclusions, with necessary critical reservations, may be drawn from a study of the observations made on color phenomena in the visible region with heterochromatic light:

(1) Derivatives of *p*-nitrophenylhydrazine containing a second nitro

<sup>11</sup> The compounds marked with an asterisk are new and their M.P.s are recorded. The preparations were made as usual, but it is advisable to heat the mixtures of the respective components for longer periods in the case of dicarbonyl compounds. The derivatives of the di- and tri-carbonyl substances proved, on analysis, to be bis-hydrazone.

*Mono- or bis-o-nitrophenylhydrazones*

Compound	Color	M.P. °C.	Color in sodium ethylate solution
<i>m</i> -Nitrobenzaldehyde	red	230	violet red (warm)
<i>p</i> -Nitrobenzaldehyde	deep red	255	blue violet
<i>m</i> -Nitroacetophenone*	red	197	yellow
Glyoxal	red	295	violet

*Mono- or bis-m-nitrophenylhydrazones*

		°C.	
<i>m</i> -Nitrobenzaldehyde	orange	213	brown
<i>p</i> -Nitrobenzaldehyde	red	228	indigo blue
<i>m</i> -Nitroacetophenone*	yellow	200	yellow
Glyoxal	orange	299-300	yellowish

*Mono- or bis-p-nitrophenylhydrazones (PNH)*

		°C.	
<i>o</i> -Nitrobenzaldehyde	red	237-258	permanganate red
<i>m</i> -Nitrobenzaldehyde	orange	247	permanganate red
<i>p</i> -Nitrobenzaldehyde	deep red	248	indigo blue
<i>m</i> -Nitroacetophenone*	yellow	247	violet
Glyoxal	red	308	indigo blue
Diacetyl	orange	310	indigo blue
Acetylcaproyl*	deep red	271	indigo blue
Acetonylacetone*	orange	210	orange red
Furil*	yellow	208	deep violet
Benzil	orange	192	deep violet
Benzaldehyde†	orange	192	violet

† In sharp contrast to this combination, the phenylhydrazone of *p*-nitrobenzaldehyde (deep red, m.p. 159-160°C.) produces an olive-green color in sodium ethylate solution.

*Mono- or bis-2,4-dinitrophenylhydrazones (DNH)*

Compounds	Color	M.P. °C.	Color in sodium ethylate solution
<i>m</i> -Nitrobenzaldehyde	yellow	268	brownish violet
<i>p</i> -Nitrobenzaldehyde	yellow	320	permanganate red
<i>m</i> -Nitroacetophenone*	brown red	250	violet red
Furfural	deep red	218	brown red
Benzaldehyde	orange red	233	red
Glyoxal	orange red	326-328	violet
Diacetyl	orange red	314-315	violet
Acetylcaproyl*	deep red	213	deep violet
Acetonylacetone*	yellow	259-260	red (warm: violet)
Phenylglyoxal*	orange red	280.5	blue violet
Acetylbenzoyl*	orange yellow	260	red violet
Diphenyltriketone*	red	271	violet
Furil*	deep red	209-210	violet

group anywhere, or in the *para* position, if benzene derivatives, form alcoholic alkali solutions of a blue color.

(2) The corresponding derivatives of *o*-nitrophenylhydrazine show a reddish color in solutions.

(3) Alkaline solutions of 2,4-dinitro derivatives, which are combinations of (1) and (2) exhibit a violet color formed by mixture of red and blue.

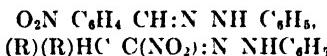
(4) The presence of a nitro group in the *meta* position shifts the color toward the yellow.

We have also tested the coloration of alcoholic alkali solutions of some *p*- and *m*-nitrobenzoylhydrazone,<sup>12</sup> and have found that these compounds, which are light-yellow, give no characteristically colored alkaline solutions. We tested the *p*-nitrobenzaldehyde *p*- and *m*-nitrobenzoylhydrazone, the *m*-nitroacetophenone *p*-nitrobenzoylhydrazone and the glyoxal *m*- and *p*-nitrobenzoylhydrazone, all of which were more or less difficultly soluble in sodium ethylate solution with a yellow or orange yellow color.

However, this is not wholly contradictory to the "rule" stated above. In these compounds the configuration is:



which is totally different from the color-producing combinations



or



In the latter cases the nitro group is present either in the molecule of the reacting carbonyl compound or in the phenyl radical directly connected with the N of the hydrazine. It thus seems plausible that the nitrobenzoylhydrazone are unable to react in the tautomeric form whose alkali salts show these characteristically deep colors.

The conduct of the method is simple. All details may be taken from the given description. For the special purpose of determining the 3 butylene compounds (2,3-butylene glycol, acetyl-methylcarbinol, and diacetyl), the following details may be stressed. For transformation of acetoin into diacetyl the concentration of  $\text{FeCl}_3$  as an oxidant should be low, as Barnicoat (53) and Tomiyashu (54) have observed destruction of diacetyl due to the iron salt. A 4% concentration of  $\text{FeCl}_3$  in the test solution is recommendable.

The determination limit for diacetyl, when the bis-DNH-compound

<sup>12</sup> The preparation of some 3-nitrobenzoylhydrazone is described by Strain (49).

is used as a colorimetric basis, is approximately ten times sharper than with the methods using the Ni- (or Fe-) compound of dimethylglyoxime (24, 25, 55).

There is an advantage in the use of this method as compared with that depending upon formation of acetaldehyde by oxidation with periodic acid (56). With this method there is no interference because of the presence of preformed acetaldehyde, alcohol, lactic acid, or, especially, sugar. The treatment with  $\text{FeCl}_3$  mentioned above takes place in a weak hydrochloric acid medium. Traces of furfural or hydroxymethylfurfural may be formed and may pass into the distillate together with the diacetyl. These substances are precipitated as DNH together with the diacetyl-bis-DNH, but can be removed by eluting the kaolin and adsorbed precipitate with 5% aqueous methanol solution. Extremely dilute solutions of diacetyl can be concentrated according to the very effective original direction of Diels and Stephan (57) by repeated distillation from a saturated  $\text{NaCl}$  solution.

Analytically pure  $\text{NaCl}$  must be used exclusively. Using table salt we were surprised to find the diacetyl value from blood or urine unprecedently high. However, the bluish color of the sodium ethylate eluates, which could not be overlooked, led to the conclusion that a considerable amount of the methylglyoxal derivative had been formed. This was due to the fact that the commercial table salt (Diamond Crystal Salt Co.) contained 0.04% dextrose, 0.05%  $\text{NaHCO}_3$  and 0.9% tricalcium phosphate. Control tests proved that these small amounts of sugar, at the boiling temperature of a 30%  $\text{NaCl}$  solution, can produce substantial quantities of methylglyoxal. This is in conformity with the findings (58) made on the origin of this substance from sugars treated with  $\text{NaHCO}_3$  or  $\text{Na}_2\text{HPO}_4$ .

#### ACKNOWLEDGMENTS

We are indebted to Carbide and Carbon Chemical Corporation, Commercial Solvents, the Lucidol Corporation, and Sharples Chem. Inc., for placing various chemicals at our disposal.

The authors also wish to express their appreciation to the Ella Sachs Plotz Foundation for the Advancement of Scientific Investigation and to Swift & Co., Inc., Chicago, Ill., for the award of a grant.

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## Biochemistry of Wound Healing

### I. The Thiamine Content of Healing Tissue of Skin Wounds

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Received March 12, 1945

#### INTRODUCTION

The vitamin content of various tissues has received a great deal of study during the past few years. It has been postulated (1) that a study of the vitamin content of tissues may throw light on their metabolism.

The vitamin content of healing tissue has not been extensively investigated. Uzbekov (2) has found that in normally healing wound tissue the vitamin C content is equal to that of healthy tissue but that in slowly healing tissue the C value approaches the scorbutic level. Bartlett, Jones, and Ryan (3) analyzed abdominal wounds of guinea pigs on high and low vitamin C diets. The vitamin C content of the wounds varied with the vitamin C intake. In animals on a scorbutic diet the ascorbic acid in the healing area was not increased over control biopsies. In animals on a high ascorbic acid intake the content in the healing wound was much greater than in the control biopsy. We have found no data on other vitamins in healing tissues in the literature.

It was believed that some understanding of the metabolism of wound repair might be gained by a comparative study of the vitamin content of normal and healing tissue. The universal dependence of living cells on an adequate supply of thiamine and the apparent universality of function of thiamine led us to select this factor for a preliminary study in the biochemistry of wound healing.

This paper deals with the *thiamine* content of repair tissue, as measured by the fermentation method, in normally healing skin wounds on adult female *albino rats* at various stages of healing.

## EXPERIMENTAL

### *Animal*

It was our desire to work with rapidly healing untreated wounds. Experience has shown that when ordinary precautions for cleanliness are exercised, skin wounds of the albino rat remain relatively free from infection.

Healthy mature females which had been reared throughout their entire life on the colony diet of commercial dog foods supplemented twice weekly with fresh lettuce and carrots were selected. These animals were in the latter half of the life span ranging in age from 514 to 637 days (average = 555 days) and in weight from 250 to 350 grams. They were distributed into groups as uniformly as possible with regard to age, weight, and general condition. The groups consisted of animals with standard wounds allowed to heal for periods of 3, 6, 9, 12, and 18 days respectively.

Wounds were made under ether anaesthesia in the freshly shaved and washed shoulder region. A sharp sterile No. 7 cork borer was rotated against the skin to produce a mark. The center of this area was picked up with rat-tooth forceps and the skin excised with scissors to the depth of its natural separation from underlying tissue following the guide lines produced by the cork borer. This method produced reasonably uniform wounds about 1 centimeter in diameter. No antiseptic was used on the wound at any stage of the experiment.

Measurements of the wounded area on each animal were taken at the time of wounding and at intervals throughout the experiment. Area was calculated from the average of three diameters taken with calipers (4, 5). The scab was left undisturbed during measuring.

At the pre-determined stages of healing, animals were killed by ether anaesthesia. A control section of skin of 200 mg. to 300 mg. in weight was removed from the shaved hip area opposite to the shoulder wound. This area was chosen for control determinations in order that the sample might be taken as far from the wound site as possible to eliminate possible local changes due to wounding or healing.

The sample from the wound area was taken by first removing the scab and then excising only the healing granulation tissue underneath. At the 18 day or completely healed stage the repaired newly epithelized tissue as evidenced by a shiny white scar area was excised and analyzed.

*Analytical*

Since the tissue weights were very small and of low potency the Atkin, Schultz, and Frey (6) ultramicro method for thiamine determination was chosen as the most suitable for this type of sample. The adaptation of Carleen and co-workers (7) who were using muscle biopsy samples in a range of 0 to 5 millimicrograms of thiamine was further modified for our use. Good correlation (8, 9, 10) between the thiochrome method, the rat-curative method, and the yeast fermentation procedure has been reported.

Tissues were weighed as soon as removed from the animal, ground, and transferred to 50 ml. Erlenmeyers with 10-20 ml. of water adjusted to pH 3.0 with H<sub>2</sub>SO<sub>4</sub> (11). The flasks were steamed for 20 minutes, cooled, centrifuged, and the extract made to volume (usually 25 to 50 ml.) after adjusting the pH to 6.0-7.0 with N/10 NaOH. Aliquots were used for determining total activity and for sulfite treatment.

The sulfite procedure as described by Schultz, Atkin, and Frey (11) was modified for micro samples by using 25 mg. of Na<sub>2</sub>SO<sub>3</sub> in a 10 ml. aliquot and by using 0.3% H<sub>2</sub>O<sub>2</sub> for destroying the excess sulfite. A known amount of thiamine was added to the solution after sulfite treatment so that the test aliquot contained at least 1 millimicrogram of thiamine. The sodium ion added during the sulfite treatment was compensated for in the thiamine controls and in the non-sulfited sample by adding a similar calculated amount of Na<sub>2</sub>SO<sub>4</sub>. The flasks containing the final test solutions had been previously wetted with a 1% gelatin solution as described by Josephson and Harris (8).

Deutsch (12) has investigated the effect of substances other than thiamine on the fermentation procedure and finds some stimulation from Na<sub>2</sub>SO<sub>4</sub>-split thiamine. In our sulfiting procedure we followed the recommendation in the literature (11) of adding a known amount of thiamine to the sulfited sample. Therefore, our measurements were still in the 1 to 5 millimicrogram range. This procedure was apparently not followed by Deutsch and might account for some of the discrepancies between his results and those of other workers.

The conventional Warburg apparatus provided with 15 ml. flasks with side-arm vent was used in our experiments. The samples containing 1 to 5 millimicrograms of thiamine were added as 2 ml. aliquots of aqueous solutions. Each flask contained 1 mg. of yeast in 1 ml. of medium prepared according to Atkin, Schultz, and Frey (6). The apparatus was flushed with nitrogen for 1 hour and CO<sub>2</sub> production was measured during the second hour.

The thiamine content of the wet tissue was calculated by correcting the total fermentation-stimulating activity of the sample for the sulfite blank.

## RESULTS

The average values for the thiamine content of rat skin are fairly consistent for the five groups of animals. We have found no value for the thiamine content of rat skin in the literature. The range in values which we obtained in 26 determinations on rat skin from the hip area was from 0.11 to 0.98  $\mu$ g. of thiamine per gram (average = 0.57).

Skin removed from the shoulder at the time of wounding was analyzed in two cases in order to eliminate choice of site as a factor in the difference of thiamine content of healing tissue over normal skin. These two samples gave values of 0.42 and 0.47 micrograms of thiamine per gram of skin, well within the range obtained in the hip area and definitely lower than healing tissue values.

The results of the analyses for thiamine content of repair tissue and normal skin at different stages of healing are summarized in Table I with their standard deviations. In Fig. 1 the ratio of the thiamine content of repair tissue to normal tissue has been plotted

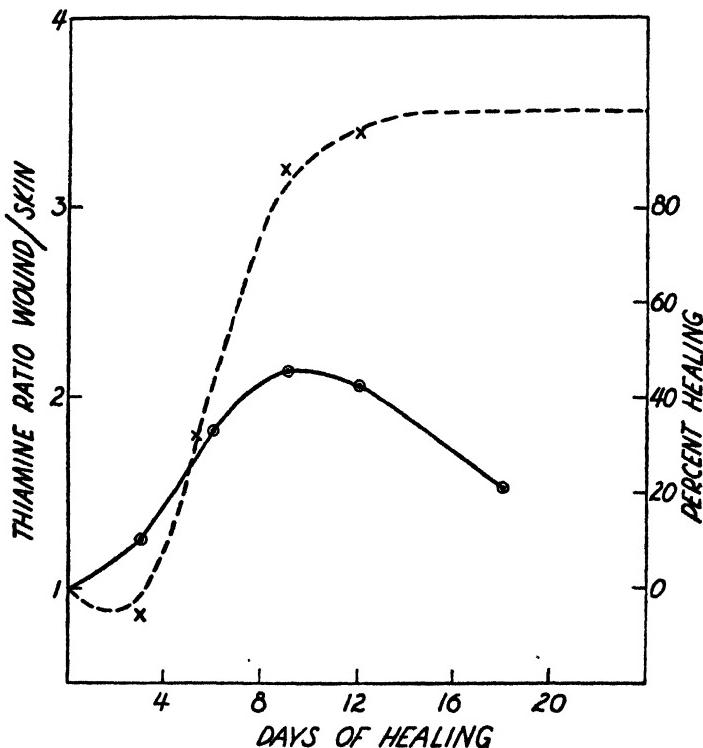


FIG. I

Relationship between Thiamine Content of Repair Tissue and Rate of Healing

Thiamine ratio  $\frac{\text{wound}}{\text{skin}}$

Per cent of the wound area healed

against days of healing. On the same graph has been plotted the percentage of wound healing against time. From these curves it is apparent that the increased thiamine content of the tissue occurs during the period of rapid healing.

### DISCUSSION

From Table I it is apparent that the variation encountered in these determinations was large. However, of the twenty-six animals

TABLE I

*Thiamine Content of Normal Skin and of Healing Tissue of Skin Wounds*

Stage of healing days	No. of rats	Av. age of rats days	Av. sample Wound Wt. mg.	Skin μg. per gram	Wound μg. per gram	Wound Skin ratio
3	5	553	117	.56±.07	.70±.12	1.25
6	6	573	83	.52±.25	.94±.56	1.81
9	6	548	21	.51±.23	1.09±.25	2.14
12	7	561	16	.70±.19	1.45±.40	2.07
18	4	530	45	.56±.14	.87±.41	1.55

tested, in twenty-three the thiamine in healing tissue was higher than in normal skin. Therefore, the difference between wound and skin thiamine in each animal of each group was subjected to statistical analysis by applying Student's t value (13). This treatment reveals that at the 3 day and 18 day stages the increases over the skin value are not highly significant ( $P = 0.4 - 0.3$  in each case). From Fig. 1 it may be seen that neither of these periods coincides with rapid wound healing as determined by decrease in area. The increases in thiamine at the 6, 9, and 12 day periods are statistically significant ( $P = 0.05 - 0.02, < 0.01, 0.05 - 0.02$ , respectively) and, in contrast to the 3 and 18 day periods, occur during the stage of rapid healing.

In connection with correlation between stage of healing and thiamine content it is of interest to report here on a single animal not included in the above groups in which the wound area became infected. This wound was analyzed at the 12 day period. The wound was less than 50% healed at this time whereas wounds of the normal animals of the 12 day group were 92-98% healed. The thiamine content of the infected wound was 0.31 μg. per gram and that of the skin 0.76 μg. per gram. This is the reverse of our findings in rapidly healing tissue. An investigation of the thiamine content of rapidly healing wounds

as compared with that of infected wounds in a series of animals would be of interest.

Sulfite blanks on skin and especially wound samples were necessary to rule out the possible stimulation of yeast by non-thiamine substances. We found no significant difference between skin with an average of 22.4% sulfite stimulation and wound with an average of 28.3%. These values compare favorably with those previously found in natural materials (8, 11, 14).

The true significance of the increased thiamine content of healing tissue over normal skin in terms of metabolic processes can not yet be stated. Williams (1) has found no correlation between thiamine content and rate of oxygen uptake by tissues nor between thiamine content and anaerobic glycolysis. However, he was comparing tissues from different organs of the body. The increase in the rate of oxygen consumption of healing tissue has been noted by v. Gaza and Gissel (15) and also by Fardon and co-workers (16). Preliminary experiments in our own laboratory also indicate a  $Q_{O_2}$  of repair tissue higher than that of normal skin. Increase in the rate of anaerobic glycolysis of healing tissue has also been reported (15). Further investigation will be necessary to determine whether the increased thiamine content of healing tissue has any relation to the increase in glycolytic activity or the increased oxygen uptake.

Experiments (5) in which the effect of local application of ointments containing vitamin B have been studied show no effect on rate of wound healing. In wounds of this nature in which the blood supply has not been seriously affected, the increase in total thiamine required should be readily supplied by the blood stream.

#### SUMMARY

1. The thiamine content of repair tissue in skin wounds at the stage of rapid healing is approximately double that of normal skin.
2. The average thiamine content of normal rat skin has been determined to be 0.57  $\mu$ g. per gram in animals in the latter half of life.

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# The Distribution and Comparative Content of Certain B-Complex Vitamins in Pork Muscular Tissues<sup>1</sup>

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Received April 9, 1945

## INTRODUCTION

It is well known that the vitamin contents of cuts of meat vary greatly, even among different animals of the same species. These variations from animal to animal may be due to differences in the quality of the feed consumed (1, 2, 3, 4, 5) but they are apparently not associated with physiological or metabolic differences. On the other hand, certain species have characteristic tissue vitamin contents which differentiate them from other species. Thus, pork is recognized to be an outstanding source of thiamine as compared to other meats. Such variations between species may represent differences in physiological mechanisms.

Wright, *et al.* have shown that great differences exist among the various organs of a single animal (6). Other investigators (7, 8, 9) have shown that muscle tissue from a single animal may vary in vitamin content depending upon the location sampled. Such variations are reflected in the different values reported for adjacent cuts of meat (5). These differences in the vitamin content of the various parts of the skeletal musculature suggest variations in the metabolic systems involved. Despite this, there has been no systematic survey of the extent of these differences in the vitamin levels for muscle samples from one animal except for studies of the riboflavin content of beef and pork muscles (8, 9).

Believing that such a study might provide information of both

<sup>1</sup> Part of this material was presented April 13, 1943 before the Division of Biological Chemistry at the American Chemical Society meeting in Detroit, Michigan.

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practical and scientific interest, the following investigation was conducted in an attempt to determine the extent of the variations of the vitamins of the B-complex in muscles from individual pigs.

### EXPERIMENTAL

Fourteen to 24 muscles from each of four sides of pork have been studied. The first three animals were obtained directly from a commercial pork processing plant within an hour after dispatch and were dissected immediately. The fourth side of pork was obtained from an animal which had received a diet supplying an excess of thiamine (50 mg. per day).<sup>3</sup> Muscles dissected from it were packed in dry ice for transport to the laboratory.

After excess fat and tendons had been trimmed from the muscles selected for analysis, the samples were ground three times, with thorough mixing after each passage through the hasher. Portions of these ground muscles were taken for moisture, protein, and vitamin analyses, for pH determinations, and for color rating. The remainders of the samples were immediately frozen at -20°F. and held at that temperature until needed for further analyses.

In preparing solutions for vitamin assay, a 100 g. quantity of each sample was homogenized with 200 g. of water in a Waring Blender and aliquots of the blends were weighed into the extraction flasks.

Thiamine contents of the samples were determined by one of two modifications of the thiochrome procedure. The first method, essentially that of Conner and Straub (10), entailed acid and enzymatic digestion of the sample, purification of the extract by adsorption on and elution from Decalso, and conversion of the thiamine to thiochrome. The second modification was identical with the first except that the adsorption and elution step was omitted, thus corresponding closely to "Method 3" of Hennessy and Cerecedo (11). When using the first method the thiamine standard was made up to volume with acid potassium chloride solution but when the adsorption and elution steps were omitted the standard was diluted with distilled water. The latter standard consistently yielded 5-8% less fluorescence than that prepared in acid potassium chloride. Since both modifications gave similar analytical values ( $\pm 5\%$ ) with the first series of samples (and in numerous other muscle analyses) the second method was used thereafter.

Riboflavin, niacin, and pantothenic acid were determined microbiologically upon enzyme digested aliquots of the samples (Cheldelin, *et al.*, 1942, 12). In addition, the riboflavin in the samples from animal 4 was determined by the fluorometric method of Peterson, Brady, and Shaw (8). Deviations of the fluorometric values from the microbiological ranged from +14 to -11%, with an arithmetic average of +2%.

Protein and moisture values were obtained by methods essentially those of the Association of Official Agricultural Chemists. Fat free tissue has been considered to be the sum of the moisture and protein, since these two components plus fat account for over 99% of pork skeletal muscle.

<sup>3</sup> We are indebted to Dr. Russell Miller for samples from one of the animals used in his study of the effect of thiamine intake on the thiamine content of pork (4).

Measurements of pH were made on the freshly ground samples, using a Beckman pH Meter. Color was estimated by eye, the samples being arranged in order of increasing redness by three different observers.

Each vitamin value reported represents the average of at least two analyses checking within  $\pm 10\%$  of the mean value for the sample. Most checks were well within  $\pm 5\%$ . Tables I and II present the data collected.

### DISCUSSION

Since fat contains relatively small amounts of the B-complex, calculations have been made on a fat-free basis, thereby avoiding the "dilution" effect of fat. However, the magnitude of the difference in vitamin content of the muscles is so great that the picture would not be changed significantly if the vitamin contents were expressed in terms of the muscles as sampled. For the most part, fat-free tissue accounts for 90-95% of the samples (Table I). The flank muscles show lower values owing to the inclusion of small amounts of surface fat.

The variations in vitamin content are large and apparently are consistent from animal to animal (Table II).

It must be realized that the averages of the values do not represent the average vitamin content of the fat-free carcasses since there has been no adjustment made for differences in the weights of the muscles. The average values, shown in Table II, are for purposes of comparison only and merely indicate whether the carcasses contained relatively little or much of the vitamins.

By inspection of Table II, it may be seen that the muscles relatively rich in thiamine in animal No. 1, are also rich in that vitamin in animals No. 2, 3, and 4 (*e.g.*, the longissimus dorsi, the gluteus profundus, and the semimembranosus are rich in thiamine in each of the four animals, while the brachialis, the internal oblique, and the deltoid are representative of the consistently less potent muscles). Animal No. 4, which received an excess of thiamine, shows a distribution similar to that of the other three. Thus, even in the presence of an abundant supply of thiamine, the muscles store varying amounts. This must reflect physiological differences in the thiamine requirements or in the storage capacities of the muscles.

Niacin shows variations similar to those of thiamine, some muscles being one and a half to two times as potent as others. For the most part, muscles rich in thiamine are also rich in niacin.

TABLE I  
*Proximate Analysis, pH, and Color of Various Pork Muscles*  
 Animal No. 2

General Location	Muscle	Protein	Moisture	Fat-Free Tissue	pH	Color*
Ham Muscles	Biceps femoris	20.56	72.3	93.0	5.53	14
	Semitendinosus	20.63	72.5	93.1	5.58	9
	Semimembranosus	22.19	73.4	95.6	5.57	19
	Gastrocnemius	21.31	73.3	94.6	5.51	16
	Gluteus medius	20.56	71.6	92.2	5.49	1
	Tensor fasciae latae	22.50	73.9	96.4	5.66	24
	Gracilis	21.25	73.2	94.4	5.43	10
	Rectus femoris	21.31	74.4	95.7	5.59	22
	Gluteus profundus	21.25	72.5	93.7	5.49	12
	Obturator externus	20.75	73.2	93.9	5.58	23
Shoulder Muscles	Deltoid	21.00	72.6	93.6	5.41	13
	Biceps brachii	21.25	72.9	94.1	5.53	15
	Extensor carpi radialis	22.19	73.0	95.2	5.60	11
	Brachialis	21.94	74.6	96.5	5.70	21
	Anterior deep pectoral	19.56	74.7	95.3	5.68	17
Flank Muscles	Posterior deep pectoral	19.56	69.9	89.5	5.40	8
	Latissimus dorsi	19.88	66.0	85.9	5.47	6
	Rectus abdominis	19.25	67.0	86.2	5.65	7
	External obliquus	19.19	66.5	85.7	5.66	5
	Internal obliquus	20.13	74.2	94.3	5.63	18
	Transversus abdominis	19.88	71.0	90.9	5.83	20
Loin Muscles	Longissimus dorsi, ant.	21.31	72.6	93.9		4
	Longissimus dorsi, cent.	22.31	71.9	94.2	5.57	2
	Longissimus dorsi, post.	22.13	72.1	94.2	5.52	3

\* Numbers denote increasing redness, 1 being least red and 24th the reddest.

Differences of similar magnitude exist in the muscle contents of riboflavin and pantothenic acid. With these vitamins the tendency for any given muscle to be high, intermediate or low in all the animals is less consistent; there being a number of samples which are relatively low in one animal but much higher in the others. For the most part, however, those muscles which are rich in riboflavin in one animal are also rich in riboflavin in the other animals. It may also be observed

TABLE II  
*Distribution of Vitamins of the B-Complex in Pork Muscles*

No.	Muscle	Micrograms of vitamins per gram of fat free tissue											
		Thiamine				Riboflavin				Niacin			
		Animal Number		Animal Number		Animal Number		Animal Number		Animal Number			Pantothenic Acid
		1	2	3	4	1	2	3	4	1	2	3	4
1	Biceps femoris	13.3	7.0	11.7	15.5	2.9	3.3	3.7	1.4	85	75	64	46
2	Semitendinosus	13.6	7.0	10.7		2.9	3.4	3.5		73	68	46	5.3 4.7
3	Semimembranosus	11.9	8.2	12.4	18.4	3.3	3.6	3.3	1.3	93	78	64	60 3.9 4.8 4.1
4	Gastrocnemius	9.4	5.2	8.5	13.1	2.9	3.3	2.2	2.0	78	68	62	60 6.0 5.6 3.5
5	Gluteus medius	11.5	6.7	10.7		2.1	2.3	2.1		85	71	47	5.3 4.1
6	Tensor fasciae latae	11.8	5.8	13.2		3.1	3.7	2.0		82	65	71	4.2 6.6
7	Gracilis	12.5	6.2	9.8	15.1	2.6	2.8	2.5	1.3	83	67	50	56 6.0 4.7 4.0
8	Rectus femoris	10.6	7.5	9.4	16.3	3.2	3.6	2.4	3.1	75	71	66	53 6.9 6.1
9	Gluteus profundus	15.3	9.1	12.7	17.6	2.4	4.2	2.2	1.2	90	78	74	47 3.1 4.1
10	Obturator externus	11.1	6.4	7.1	11.2	3.4	5.4	3.7	2.9	64	75	45	45 7.7 9.1
11	Deltoid	9.7	6.4	6.6	14.6	3.4	3.6	3.3	1.7	61	74	43	43 10.0 5.9 4.3
12	Biceps brachii	10.8	6.4	8.1		2.7	3.4	4.6		65	80	46	7.3 6.4
13	Extensor carpi radialis	10.5	5.8	7.7		2.6	3.2	2.8		64	68	36	5.9 4.9
14	Brachialis	0.5	2.8	4.4	5.7	2.2	2.9	3.0	1.4	65	63	42	58 9.2 7.0
15	Posterior deep pectoral	12.5	7.4	11.3	17.9	2.6	3.1	3.4	1.7	55	74	51	55 6.5 5.8 3.8
16	Anterior deep pectoral	8.2	5.2	9.6		3.8	3.8	3.0		84	58	53	10.0 7.7
17	Latisimus dorsi	13.2	7.6	11.3	13.9	2.8	3.2	3.0	1.6	85	77	57	58 7.0 6.0 3.8
18	Rectus abdominis	11.8	6.7	7.3	13.6	2.6	3.0	2.7	1.8	84	74	52	51 7.8 7.3 5.7
19	External obliquus	13.5	6.6	8.3		2.2	3.0	2.6		76	80	49	7.0 6.9
20	Internal obliquus	10.3	5.5	6.3		3.6	3.5	2.9		67	68	59	5.5 8.0
21	Transversus abdominis	11.7	6.0	8.2		2.7	3.6	3.2		61	72	52	7.3 7.6
22	Longissimus dorsi, ant.	13.6	9.6	12.7		2.1	2.4	1.7		98	63	70	3.3 5.5
23	Longissimus dorsi, cent.	14.5	8.0	13.3	17.7	2.1	2.3	1.5	1.0	90	66	78	37 3.1 4.2
24	Longissimus dorsi, post.	16.8	8.4	12.3		2.1	2.4	1.8		94	66	75	3.1 4.2
	Avg.	11.8	6.7	9.7	14.5	2.8	3.3	2.8	1.8	77	71	56	52 6.2 6.0 3.9

that these same muscles tend to be rich in pantothenic acid. Moreover, those muscles which contain much thiamine and niacin are usually the ones containing relatively small amounts of riboflavin and pantothenic acid. Thus, there appears to be a direct correlation between the thiamine and niacin contents, and between the riboflavin and pantothenic acid contents, but an inverse correlation between these two groups of vitamins.

The riboflavin values are in general agreement with the data for pork (Peterson, Brady, and Shaw, 7) and with that for beef (Brady, Peterson, and Shaw, 8), these investigators finding ham and shoulder muscles to be richer in riboflavin than loin.

In connection with the correlations observed among the vitamins, it is interesting to note that, in humans, injection of either riboflavin or pantothenic acid increases the blood level of both riboflavin and pantothenic acid (13). On the other hand, an inverse relationship

between riboflavin and thiamine has been noted, Singher *et al.* finding that the riboflavin content of rat livers was higher in thiamine-deficient animals than in normal livers and that the thiamine content of livers from riboflavin-deficient animals was higher than usual (14). This has been confirmed by Luecke and coworkers (15). It has also been shown that intravenous injections of either niacin or thiamine will lower blood sugar, but that simultaneous injections of each will raise the blood sugar level (16).

These and many similar reports of this nature tend to indicate the existence of supplementary (or perhaps even antagonistic) enzyme systems such that changes in one system will be reflected by shifts of the others. If this is the case, the data presented in this paper may well indicate that several systems occur in widely varying ratios. Thus the low riboflavin and the high thiamine content of loins may indicate the predominance of an enzyme in which thiamine is the prosthetic group in the metabolism of that muscle; whereas the high riboflavin and low thiamine content of the brachialis would indicate an enzyme utilizing riboflavin to be carrying on a larger share of the metabolic process. The metabolism of muscles with intermediate amounts of both vitamins would be more nearly equally divided between the two or more systems.

One might expect the function or the activity of the muscles to be associated with differences of this nature, but attempts to correlate vitamin content with activity, color, or pH have not been too successful (Table I). For the most part, however, muscles subject to repeated contraction such as the brachialis, deltoid, and gastrocnemius are lower in thiamine and niacin, but richer in riboflavin and pantothenic acid than are muscles subjected to less activity, such as the loin. On the other hand, the biceps femoris and the semimembranosus muscles, both of which are used in walking or standing, are similar to the longissimus dorsi in vitamin content. However, it is difficult to assess the extent of the activity to which a muscle is subject. Perhaps the loin and the biceps femoris, although not contracted to any great extent, are under more or less constant tension while the active muscles such as the brachialis and gastrocnemius undergo marked contraction and extension during walking but are more relaxed at other times. It may be that the type of activity, *e.g.*, a constant tension as compared to spasmodic efforts, is the significant factor in determining the enzyme systems in the muscles.

From a practical standpoint, the variations in vitamin content indicate the need for great care in the sampling of meats. Small portions of a cut of meat cannot be relied upon to give vitamin data characteristic of the whole piece but the entire piece must be ground and sampled if representative values are to be obtained. In studies involving cooking or processing losses, the need for carefully paired samples is obvious.

### SUMMARY

The thiamine, riboflavin, niacin, and pantothenic acid contents of 24 pork muscles have been determined for each of several animals.

It has been shown that the vitamin content of the muscles in a single animal vary as much as 200-300%. Muscles which are high in a vitamin in one pig tend to be high in other animals of this species.

Muscles containing relatively much thiamine usually contain high levels of niacin but relatively low levels of riboflavin and pantothenic acid. Although there are indications that the activity or function of the muscle may be responsible for the variations in vitamin content, no definite conclusions to this effect can be drawn on the basis of the present data.

The theoretical and practical implications of the data have been discussed.

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# Stereochemical Configuration and Provitamin A Activity

## IV.\* Neo- $\alpha$ -carotene B and Neo- $\beta$ -carotene B

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Received January 9, 1945

### INTRODUCTION

Recently we have compared the provitamin A-activities of three well crystallized and relatively stable carotenoid pigments possessing *cis* configurations, *viz.*, neo- $\alpha$ -carotene U, neo- $\beta$ -carotene U, and pro- $\gamma$ -carotene,  $C_{40}H_{56}$ , with the potency of their respective all-*trans* isomers (4-6). The present paper reports on the biological effects of two rather labile pigments, neo- $\alpha$ -carotene B and neo- $\beta$ -carotene B, which are adsorbed below the corresponding ordinary carotenes in the Tswett column. As pointed out elsewhere (13-15) the most probable configurations of these di-*cis* pigments are characterized by the presence of one centrally located and one peripherally located *cis*-double bond in each case. The bending in the middle region of such molecules seems to be responsible for the decreased stability of their configuration and for the increased tendency to revert into the all-*trans* form.

Neo- $\beta$ -carotene B (13) is doubtlessly the main constituent of "pseudo- $\alpha$ -carotene" which was first prepared from  $\beta$ -carotene in the important study of Gillam and El Ridi (8). An analogous pigment, obtained from  $\alpha$ -carotene, *viz.*, "neocarotene," was detected by Gillam, El Ridi, and Kon (9). We find that it is difficult to isolate "pseudo- $\alpha$ -carotene"

\* For Parts I-III, see References 4-6.

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crystals which are chromatographically homogeneous. On the other hand, the above authors report that the crystals obtained from their "neocarotene" solutions were "quite different in spectral absorption from the mother liquor."

Since neo carotenes may be of nutritional importance (cf. 1), we describe below a simple procedure for testing relatively labile stereo-

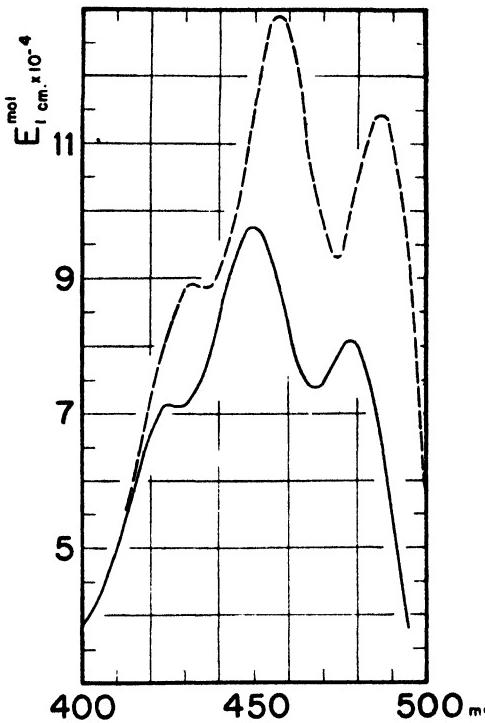


FIG. 1

Molecular Extinction Coefficients of Neo- $\alpha$ -carotene B (full line) and All-trans- $\alpha$ -carotene (dashed line) in Wesson Oil

isomers of carotenoids in quantitative bioassays without resorting to a previous crystallization. The chromatographic zones of neo- $\alpha$ -carotene B and neo- $\beta$ -carotene B can be eluted unchanged by ether. The solvent can then be removed *in vacuo* and the residue can be taken up in Wesson oil. After standardization, we administered such solutions directly to rats. Each of these operations was checked spectrophoto-

metrically to ascertain that the initial configuration remained unaltered. In the cold, the configuration of neo- $\alpha$ - or neo- $\beta$ -carotene B in Wesson oil was found to be practically unchanged for at least two weeks. This is confirmed by the fact that in the oil each of the two neo B compounds has its main extinction maximum at about 8 m $\mu$  shorter wave length than that of the corresponding all-trans compound (Figs.

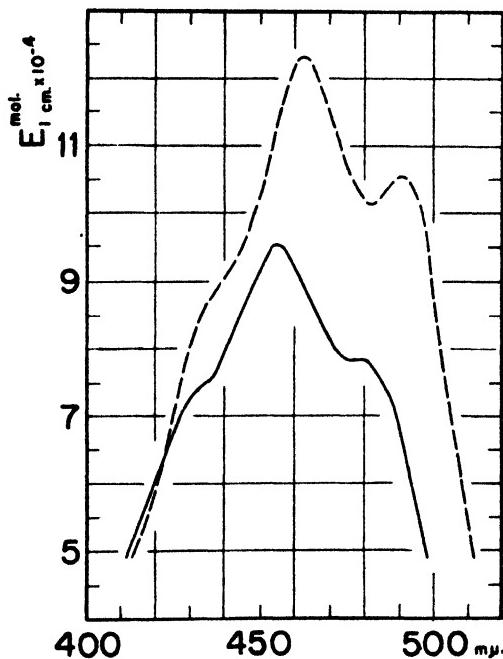


FIG. 2

Molecular Extinction Coefficients of Neo- $\beta$ -carotene B (full line) and All-trans- $\beta$ -carotene (dashed line) in Wesson Oil

1 and 2). Such differences are comparable with those which are observed in the usual solvents.

So far as we know, no detailed directions have been published concerning the administration of labile neo compounds, on the basis of which the preservation of the original configuration could be guaranteed. Some data were, however, given for a neocryptoxanthin by Fraps and Kemmerer (7).

Gillam, *et al.* (9) reported that neocarotene (mainly neo- $\alpha$ -carotene

B), "is definitely biologically active" (4 rats). Neo- $\beta$ -carotene B (the "neo- $\beta$ -carotene" of some authors) was found by Gillam to have a potency "of the same order" as  $\beta$ -carotene (8 rats). According to Beadle and Zscheile (2) it shows "significantly less" A-activity than  $\beta$ -carotene and, according to Kemmerer and Fraps (11), it has "one half the potency of  $\beta$ -carotene." In contrast, Mann's remark (12) that "the presence of pseudo- $\alpha$ -carotene in  $\beta$ -carotene solutions does not lessen the biological activity of the solution" is open to criticism.

## EXPERIMENTAL

The concentrates used for the bioassays were prepared as follows. A solution of 25–30 mg. of  $\alpha$ - or  $\beta$ -carotene in petroleum ether (b.p. 60–70°) was catalyzed with about 0.25 mg. of iodine, illuminated (see below) and developed on a calcium hydroxide column (26 × 5.8 cm.), first with petroleum ether and then with the same solvent containing 0.5 to 1% acetone, until a good separation of the stereoisomers appeared. The uppermost and lowest sections of the neo B zone were discarded, and its main portion was eluted with peroxide-free ether. This solution was dried with sodium sulfate, and rapidly evaporated at 20°C. *in vacuo*. The residue was immediately dissolved in Wesson oil (purified cottonseed oil; Wesson Oil and Snowdrift Sales Co., New Orleans). Such concentrates were made up every ten to fourteen days and were kept in darkness at 4°C. under carbon dioxide. The partially frozen supplements were thawed at room temperature every day, immediately prior to feeding. A check in the Beckman photoelectric spectrophotometer showed that the positions and the heights of the maxima remained practically constant.

In order to obtain colorimetric standardization, experiments as just described were conducted on a smaller scale. From the ether eluate of a neo B zone, four 5-ml.-samples were pipetted out, each of which was rapidly evaporated *in vacuo*. Controls showed that this evaporation did not alter the height or the position of the extinction maxima. Two of the four residues (each containing about 100 µg. of pigment) were separately dissolved in 25 ml. portions of hexane and, after catalysis with 1 to 2 µg. of iodine and illumination at 22°C. for five min. (two fluorescent Mazda lamps, 3500°, 40 W, length of tube, 120 cm., distance from the solution, 60 cm.), they were used to determine the pigment concentration on the basis of the iodine equilibrium extinction curves (13). Each of the two other residues was dissolved in 25 ml. of Wesson oil, in order to obtain the extinction maxima or the whole molecular extinction curves of the neo B compound as represented in Figs. 1 and 2.

Since the iodine equilibrium curves for the  $\alpha$ - and  $\beta$ -carotene sets were established with crystalline pigments under standardized conditions of catalysis, the possible errors are small enough for the present and many other estimations. This is not invalidated by a destruction phenomenon which is caused by iodine upon overexposure as stressed recently in a Review (14) and investigated by Zscheile, Harper, and Nash (16).

All optical readings were taken in 1-cm.-glass-cells in the Beckman apparatus. The solutions in Wesson oil were measured against pure oil. In each case several

independent experiments were carried out, on the basis of which it can be claimed that concentrations, extinctions, and constants as given are correct within a possible maximum error of  $\pm 6\%$ . The following data refer to solutions in our Wesson oil sample and may vary somewhat when other brands of this oil are used.

*Neo- $\alpha$ -carotene B:* The main maximum is located at 449–450 m $\mu$ . At this wave length  $E_{1\text{ cm.}}^{\text{mol.}} = 9.75 \times 10^4$ ;  $E_{1\text{ cm.}}^{1\%} = 1815$ ; and the concentration ( $\mu\text{g. pigment per } 0.1\text{ ml. of oil}$ ) =  $0.551 \times E_{1\text{ cm.}}$ .

*All-trans- $\alpha$ -carotene:*  $E_{1\text{ cm.}}^{\text{mol.}} = 12.9 \times 10^4$  at 457 m $\mu$ .

*Neo- $\beta$ -carotene B:* The main maximum is located at 455 m $\mu$  where  $E_{1\text{ cm.}}^{\text{mol.}} = 9.56 \times 10^4$ ;  $E_{1\text{ cm.}}^{1\%} = 1780$ . The concentration ( $\mu\text{g. per } 0.1\text{ ml. oil}$ ) =  $0.562 \times E_{1\text{ cm.}}$ .

*All-trans- $\beta$ -carotene:*  $E_{1\text{ cm.}}^{\text{mol.}} = 12.3 \times 10^4$  at 463–4 m $\mu$ .

The bioassays were carried out as reported earlier (4) in the presence of 0.5 mg. of  $\alpha$ -tocopherol<sup>1</sup> per 0.1 ml. of Wesson oil (10). All supplements were administered orally, by means of a hypodermic syringe having a blunt needle.

TABLE I

*Summary Table of Bioassay Experiments on Male and Female Rats Receiving  
All-trans- $\beta$ -carotene, Neo- $\alpha$ -carotene B or Neo- $\beta$ -carotene B in  
Cottonseed Oil or the Oil Alone (Negative Controls)*

(The average results on males and females are weighted equally. Where animals died in the course of the experiment, the number of animals still alive which is included in the average is given in parentheses. The average age at the start of the depletion period was 22–23 days.)

Supplement	Dose per day	Number of rats		Depletion period			Assay period						
				Aver- age weight at start	Aver- age dura- tion	Aver- age final weight	Average increase in body weight up to the following days						
		Male	Fe- male	g.	days	g.	5th	10th	15th	20th	25th	28th	
All-trans- $\beta$ -carotene	μg.												
	0.6	8	7	41.2	21	91.4	1.2	14.0	23.2	31.0	39.4	42.4	133.7
	1.2	9	7	42.4	21	91.4	4.9	21.4	35.4	47.3	57.0	61.5	153.0
Neo- $\alpha$ -carotene B	1.2	9	7	42.0	20	90.8	-2.4	3.2	6.8	9.0	9.3	11.1	101.2
	2.4	9	8	42.0	21	87.8	1.9	10.6	18.0	22.2	25.6	28.4	116.2
	4.8	9	7	41.8	22	90.6	3.2	17.6	30.4	41.4	47.7	50.3	141.4
Neo- $\beta$ -carotene B	1.2	9	7	42.2	21	91.5	3.3	16.1	27.1	35.8	42.1	44.2	135.7
	2.4	8	8	41.7	21	92.0	10.2	26.5	39.9	53.5	62.2	66.0	158.2
	4.8	9	7	42.2	20	89.0	10.0	27.2	42.5	54.4	62.4	66.9	156.0
Negative controls	0.0	21	12	41.5	21	89.8	0.1 (32)	0.5 (31)	0.6 (29)	-5.0 (27)	-4.4 (21)	-5.9 (16)	84.3 (16)

<sup>1</sup> This quantity was erroneously given as "0.5 μg." in our III paper, p. 160.

## RESULTS

Table I gives a summary of the data on the 162 rats used which came from our stock colony.

The 28 day weight increases for the rats receiving all-*trans*- $\beta$ -carotene were somewhat higher than those in the earlier series (5, 6). The average increases in the new present series were 42.4 g. for the group receiving

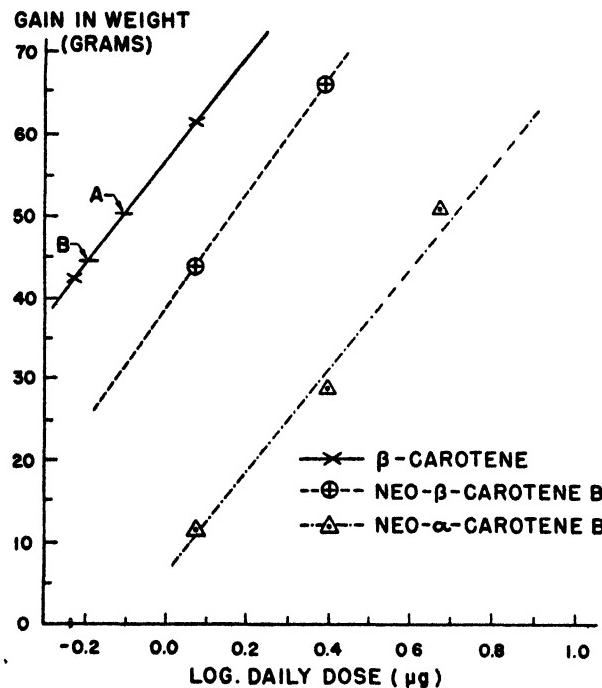


FIG. 3

The Relationship of Gain in Weight to log. of Daily Dosage of All-*trans*- $\beta$ -carotene, Neo- $\alpha$ -carotene B, and Neo- $\beta$ -carotene B

0.6  $\mu\text{g}$ . of  $\beta$ -carotene daily (compared with 25.0 and 29.5 g. in the earlier assays) and 61.5 g. for those rats which were given 1.2  $\mu\text{g}$ . daily (earlier values: 47.9 and 51.6 g.). In all these assays the litter mates were distributed as evenly as possible among the various groups. Consequently, the most probable explanation of the differences in growth just mentioned seems to be an influence of seasonal variations. We believe

that deviations of the described nature do not influence the relative potencies of carotenes as found in independent assays.

Concerning the results obtained with neo- $\alpha$ -carotene B and neo- $\beta$ -carotene B, we refer to Table I. In the negative control group, which included one rat from each of the 34 litters, 24 (or 73%) were losing weight or had died by the tenth day of the assay period while less than 50% survived for the 28 days. These survivors lost approximately 6 g.

The dosage/gain-in-weight curves are given in Fig. 3.

The curves connecting the three points for neo- $\alpha$ -carotene B and the two lowest dosages of neo- $\beta$ -carotene B (1.2  $\mu$ g. and 2.4  $\mu$ g. levels) are parallel with that for all-*trans*- $\beta$ -carotene. The uniformity of slopes of all three curves support the accuracy of the data. Only the group which received the highest dose of neo- $\alpha$ -carotene B (4.8  $\mu$ g.) and that which was given the lowest dose of neo- $\beta$ -carotene B (1.2  $\mu$ g.) were in the correct range to evaluate their bio-activity.

However, it should be noted that the increases in weight throughout the assay period are practically identical for the groups receiving 2.4 to 4.8  $\mu$ g. of neo- $\beta$ -carotene B daily. This would indicate that at the 2.4  $\mu$ g. level, the maximum possible gain already obtains. That the growth response to 2.4  $\mu$ g. does not greatly exceed the maximum effective level is indicated by the fact that the slope of the neo- $\beta$ -carotene B curve coincides with that for all-*trans*- $\beta$ -carotene. Also it is important to point out that the calculation of potency is based in this case on the point where the average gain of the group receiving the lowest level (1.2  $\mu$ g.) is projected on the curve for all-*trans*- $\beta$ -carotene.

The potency of neo- $\alpha$ -carotene B as calculated by the procedure of Coward (3) was found to be 16% of that of all-*trans*- $\beta$ -carotene. The biological activity of neo- $\beta$ -carotene B, on the other hand, was approximately three times as great as that of neo- $\alpha$ -carotene B. Neo- $\beta$ -carotene B was found to be 53% as active as its all-*trans* isomer.

## DISCUSSION

The potency of neo- $\alpha$ -carotene B is reduced to about one-third of its all-*trans* isomer and that of neo- $\beta$ -carotene B to about one-half of that of natural  $\beta$ -carotene. The value found for neo- $\beta$ -carotene B agrees with that suggested by Kemmerer and Fraps (11).

Table II gives a survey of the data which we have so far obtained on some stereoisomeric carotenes (4-6). We believe that these figures

are accurate within a maximum possible error of about  $\pm 10\%$  of each value.

Even the limited content of Table II permits the following, more general statements.

1. In conformity with observations made by earlier authors, the steric configuration of a carotenoid is as strong a determining factor of the vitamin A-activity as the structure. Indeed, the influence of the spatial situation may overrule that of certain plane-structural features.

TABLE II

*Relative Biological Activities of Some Members of Stereoisomeric Carotene Sets*  
(Nomenclature, cf. (14))

Set	Member	Probable configuration of <i>cis</i> compounds	Provitamin A potency ( $\beta$ -carotene = 100)
$\alpha$ -Carotene	all- <i>trans</i>	—	53
	neo U	9-mono- <i>cis</i> -	13
	neo B	5,9(or 6,9)di- <i>cis</i>	16
$\beta$ -Carotene	all- <i>trans</i>	—	100
	neo U	$\beta$ -mono- <i>cis</i>	38
	neo B	$\beta$ ,6-di- <i>cis</i>	53
$\gamma$ -Carotene	all- <i>trans</i> *	—	28
	pro-	3,5,7,9,11-penta- <i>cis</i>	44

\* From *Mimulus* flowers.

For example, if we could convert all-*trans*- $\beta$ -carotene into all-*trans*- $\alpha$ -carotene (by migrating one double bond out of conjugation) half of the initial bio-potency would be lost. However, a markedly stronger decrease occurs when the structure of  $\beta$ -carotene is kept unchanged while its molecules are bent around a peripheral double bond (cf. neo B).

2. It can be stated at the present time that one or two *trans*  $\rightarrow$  *cis* rearrangements diminish the bio-potency of an all-*trans* carotene. However, if the molecule recovers its generally straight shape by a greater number of such rotations, the initial A-activity may remain unchanged or even increase (cf. pro- $\gamma$ -carotene). It is not excluded that straight carotene molecules (whatever the number of their *cis* double bonds be) fit better than bent molecules into that enzyme system which is responsible for the conversion of the provitamin into vitamin A in the body.

## SUMMARY

Neo- $\alpha$ -carotene B and neo- $\beta$ -carotene B were shown to be stable for at least two weeks when dissolved in Wesson oil and kept under carbon dioxide in the dark at 4°C. The provitamin A activity of neo- $\alpha$ -carotene B was found to be 30% of that of all-trans- $\alpha$ -carotene while neo- $\beta$ -carotene B exhibited a potency which corresponded with 53% of that of all-trans- $\beta$ -carotene. Some general conclusions concerning structure, configuration, and provitamin A activity of carotenes are proposed.

*Acknowledgment.* We wish to thank Mr. A. Sandoval for his help in the chemical part of the present work.

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# On the Metabolism of *Zygosaccharomyces*<sup>1</sup>

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Received March 2, 1945

## INTRODUCTION

The total amount of acid produced by *Zygosaccharomyces acidifaciens* has been shown (Nickerson, 1943) to increase with increasing concentrations of glucose up to 30% and with increasing concentrations of ethanol up to 10%. In a 30% glucose medium, 0.080–0.088 N acid is actually produced; since the titer of the medium is about 0.02 N the cells make their environment essentially an N/10 solution of acid.

It is rather unusual for yeasts to produce more than a slight amount of acid. Pasteur, in his "Mémoire sur la fermentation alcoolique," demonstrated that succinic acid occurs normally in yeast fermentation; Ehrlich (1909) later showed this compound is derived from glutamic acid released by autolysis of yeast proteins. Hohl and Joslyn (1941) studied the production of lactic acid by seven closely related strains of *Saccharomyces cerevisiae*. Their results confirmed earlier observations that lactic acid is an important by-product in alcoholic fermentation and indicated that the lactic acid formed does not arise from amino acids but, reasoning from the parallel between the curves for acid production and sugar consumption, from the sugar fermented. Custers (1940), in a thorough study of yeasts of the genus *Brettanomyces*, found these organisms produced only acetic acid in addition to alcohol and CO<sub>2</sub> on fermentation of glucose, and that the acid is only produced aerobically. Joslyn and Dunn (1938) studied the relation of volatile acid formation to alcoholic fermentation using two wine yeasts; they found volatile acid (chiefly acetic with a little formic) formation occurring mainly during the early stages of fermentation when the oxidation-reduction potential falls rapidly.

The ability to produce acid is hardly confined to any one group of yeasts. Aerobic acid production is familiar with the widespread occurrence of film forming yeasts.

<sup>1</sup> Aided in part by a grant to the senior author from the Cyrus M. Warren Fund of the American Academy of Arts and Sciences; also, in part by a grant from the Faculty Research Fund of Wheaton College.

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Anaerobic production of lactic acid is well known, and mention of anaerobic production of acetic acid by yeasts can be found scattered throughout the older literature. The amount of acid made by some yeasts is rather high; Mrak, Phaff, Vaughn, and Hansen (1942) found *Pichia Kluyveri* produced 0.055 *N* total acid, while Kroemer and Krumbholz (1931) found *Zygosaccharomyces polymorphus* made 0.055 *N* volatile acid anaerobically. The particular species, *Z. acidifaciens*, used in this work is thus not unique in its ability to make acid anaerobically, although it does make unusually large amounts.

This paper identifies the nature and amount of acids produced by *Z. acidifaciens* together with some of the conditions for acid production. The oxidative metabolism of acetate, glucose, and ethanol is also considered. Study of glucose fermentation by this organism and the search for reduced counterparts to the oxidized metabolic products revealed the *normal* occurrence here of Neuberg's so-called 3rd type of fermentation with large amounts of glycerol and acid produced *without the aid of "steering substances."*

## EXPERIMENTAL

### Methods

Stock cultures of yeasts were maintained on wort agar (Difco) with 1% added agar. Flasks were inoculated with standard suspensions of two-day cultures grown in a basal medium consisting of: 20 g. glucose, 3.0 g. Bacto peptone, 0.1 g. yeast extract, 3.0 g.  $\text{KH}_2\text{PO}_4$ , 3.0 g.  $(\text{NH}_4)_2\text{SO}_4$ , 0.25 g.  $\text{CaCl}_2$ , 0.25 g.  $\text{MgSO}_4$ , and distilled water to make one liter. This basal medium was used in all experiments; modifications in its composition are noted wherever such exist. The organisms used were three species of *Zygosaccharomyces*: *Z. acidifaciens* Nickerson, *Z. Richteri* Lochhead and Heron, and *Z. Nussbaumeri* Lochhead and Heron. Most of the work was done with the first mentioned species. The other two species used were very kindly supplied by Dr. A. G. Lochhead.

125 cc. Erlenmeyer flasks containing 100 cc. of medium were employed; the resulting liquid depth gave approximately anaerobic conditions since all three species of yeasts used grow as a sediment only. (Joslyn and Dunn, 1938, found acid production to be practically independent of the oxidation-reduction potential using different gaseous atmospheres.) Cultures were kept at room temperature, 20–22°C. Determinations were made in triplicate on duplicate flasks in all series; there was usually good agreement among the values obtained and only averages are presented.

In time-course studies at the intervals indicated, 1 cc. samples were removed aseptically, diluted with distilled water, and titrated hot with 0.05 *N* NaOH for total acidity using phenolphthalein as indicator. Sugar remaining in the medium was determined by Benedict's (1931) method. Other analytical procedures are noted under the section on the nature of acids produced.

### Acid Production

From Fig. 1 it appears that a near proportionality exists between acid formation and glucose consumption. With the three potassium phosphates as buffers (0.3% concentration in the basal medium,

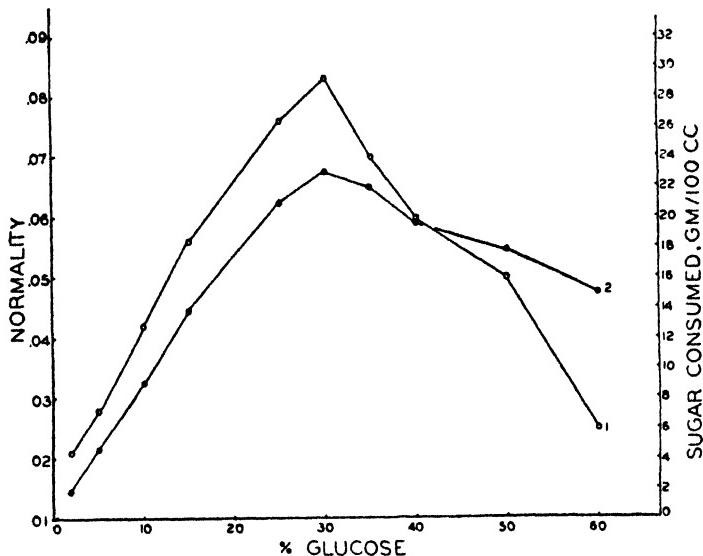


FIG. 1

Normality of acid produced anaerobically at 22°C. (open circles) by *Z. acidifaciens* in different concentrations of glucose, and amount of glucose consumed (closed circles). All cultures 15 days old when sampled; initial titer of medium subtracted.

Fig. 2), it was found that acid formation is highest starting with an acid medium. Figure 2 shows that most acid is formed with  $\text{KH}_2\text{PO}_4$  with an initial pH of 5.0 that fell to 4.0 thus suppressing the dissociation of the acetic acid formed. Table I contains similar data for two other species.

It has occasionally been mentioned in the literature (e.g., Capitain, 1930) that yeasts most tolerant of acid are those capable of producing the most acid. A series with tartaric acid added to the basal medium was studied; as Table II shows, acid formation increases with added acid up to 0.10 N, falling off beyond that concentration, growth being definitely poor beyond 0.25 N. Capitain, who studied the effect

of several organic acids, found malic acid to be most effective in stimulating acid production. While discussing acid tolerance, it is worth noting that 0.5 *N* tartaric acid, though not permitting growth, killed but a small percentage of cells within 24 hours as shown by staining with methylene blue. In fact, *Z. acidifaciens* is so tolerant of acid that the usual manometric method for determination of CO<sub>2</sub>,

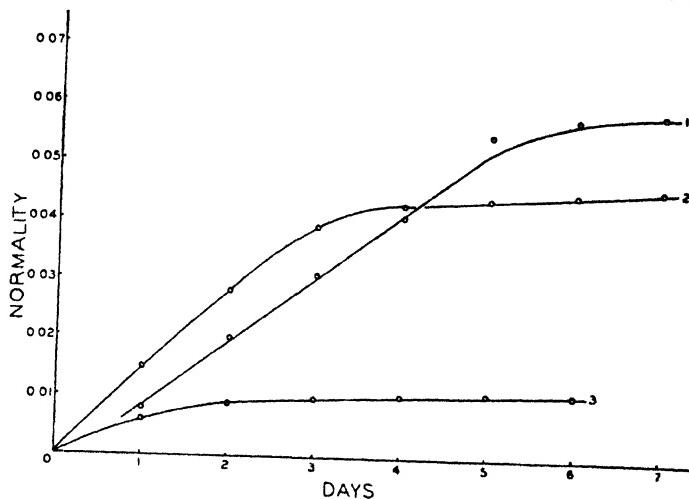


FIG. 2

Anaerobic acid production by *Z. acidifaciens* with different potassium phosphates.  
(1) KH<sub>2</sub>PO<sub>4</sub>, (2) K<sub>2</sub>HPO<sub>4</sub>, (3) K<sub>3</sub>PO<sub>4</sub>. Basal medium contained 15% glucose.

retention by adding acid could not be used since even a final concentration of 2.5 *N* HCl did not completely inhibit the glucose fermentation. Van Niel and Cohen (1942) had a similar difficulty with *Candida albicans* which required 1 *N* H<sub>2</sub>SO<sub>4</sub> to completely inhibit fermentation. Taylor (1923), studying the effect of many acids on yeast activities, found monochloroacetic acid to be by far the most toxic (and at the lowest H ion concentration). The use in manometric studies of monochloroacetic acid for stopping yeast metabolic activities might be investigated.

#### *Nature of Acids Produced*

A. *Procedures.* 750 cc. of the basal medium with 15% glucose in a 1 liter flask were inoculated with a suspension of young cells. The flasks were cotton stoppered

TABLE I  
*Total Acid Production by Species of Zygosaccharomyces*  
 15% glucose basal medium; phosphate buffers 0.3% concentration;  
 100 cc. in 125 cc. flask

Buffer	Time days	Normality—Total Acid Produced		
		<i>Z. acidifaciens</i>	<i>Z. richteri</i>	<i>Z. nussbaumeri</i>
$\text{KH}_2\text{PO}_4$	1	0.008	0.005	0.002
	2	.020	.010	.004
	3	.031	.021	.003
pH 5.0	4	.041	.035	.004
	5	.055	.038	.005
	6	.058	.039	.014
medium = $0.022 N$	7	.059	.039	.015
$\text{K}_2\text{HPO}_4$	1	0.015	0.010	
	2	.028	.020	
	3	.039	.023	
pH 6.3	4	.043	.030	
	5	.044	.034	
medium = $0.016 N$	6	.045	.034	
$\text{K}_3\text{PO}_4$	1	0.006	0.005	
	2	.009	.010	
	3	.010	.013	
pH 7.4	4	.010	.015	
	5	.011	.016	
medium = $0.009 N$				

TABLE II  
*Effect of Added Tartaric Acid on Total Acid Production  
by Species of Zygosaccharomyces*

2% glucose basal medium

Organism	Tartaric Final Cone.	pH of Medium	Total Acidity Medium	Normality—Total Acid Produced			
				2 Days	4 Days	5 Days	7 Days
<i>Z. acidifaciens</i>	0.05 N	2.8	0.093 N	0.009 N	.012 N	.019 N	.020 N
	.10	2.6	.126	.009	.023	.028	.030
	.25	2.4	.282	.007	.009	.017	.024
	.50	2.2	.513	.002	.004	.002	.002
<i>Z. Richteri</i>	0.05 N	2.8	.089	.003	.006	.008	.009
	.10	2.6	.124	.006	.007	.009	.010
	.25	2.4	.280	.002	—	—	—
	.50	2.2	.514	—	—	—	—

and incubated at 28°C. for 3 weeks when the cells were removed by centrifuging and the medium analyzed. Aliquots were titrated for total acidity. Ten cc. portions were acidified with 1 *N* H<sub>2</sub>SO<sub>4</sub>, until yellow to bromphenol blue and steam distilled in a Sellier apparatus; 90 cc. were collected and titrated with 0.1 *N* Ba(OH)<sub>2</sub>, using phenolphthalein as indicator. Duclaux determinations were made using 1 *N* H<sub>2</sub>SO<sub>4</sub> to precipitate the known amount of barium as BaSO<sub>4</sub>. After settling, 50 cc. aliquots were pipetted off, made up to 110 cc. with water and distilled.

The residue from the steam distillation was neutralized with Na<sub>2</sub>CO<sub>3</sub>, evaporated almost to dryness on a water bath, and mixed with anhydrous Na<sub>2</sub>SO<sub>4</sub> to a powder. The powder was then extracted with ether in a Soxhlet extractor for 16 hours; the ether was evaporated and the residue dissolved in water for determination of the acids. The following qualitative tests for acids were performed: pyruvic (Simon and Piaux, 1924), oxalic (CaCl<sub>2</sub>), tartaric (CaCl<sub>2</sub>), and citric (Denigès, 1899). Lactic acid was determined quantitatively by the method of Barker and Summerson (1941).

A neutral distillation on a portion of the original centrifugate was performed using a fractionating column; the first few drops were collected and tested for (1) acetaldehyde, with a few drops of 10% NaOH, (2) acetone, using cold NH<sub>4</sub>OH, in an iodoform reaction. Alcohol was determined pycnometrically in the distillate according to A.O.A.C. methods (1936).

**B. Results.** Total acid amounted to 0.056 *N*, volatile acid (consisting of acetic only) 0.04 *N*; lactic acid 0.011 *N*; and ethanol 1.5% by weight (see Table III). No pyruvic, oxalic, tartaric, nor citric

TABLE III  
*Acid Production by Z. acidifaciens from 15% Glucose-Basal Medium*

Flasks incubated 3 weeks at 28°C.

Calculation	Total Acid	Acetic Acid	Lactic Acid	Ethanol
Normality	0.056	0.040	0.011	
<i>mM/liter</i>		40	11	326
per cent of total acid	100	71	19	

acid was found. A test for succinic acid with AgNO<sub>3</sub> gave only a slight haze and was not determined quantitatively. The test for acetaldehyde with NaOH showed a faint yellowish color and gave the odor of crotonaldehyde; no acetone was found. Thus approximately 70% of the total acid is acetic and 75% of the non-volatile acid is lactic acid. To date, no acid production over the control value has been observed with *Z. acidifaciens* under strictly aerobic conditions. As acetate is so readily oxidized (see Fig. 7), it may be that technical difficulties have prevented detection of acid formed under such conditions, although, in view of the persistence of fermentation under aerobic conditions (see Fig. 5), one might expect acid production to persist.

C. *Manometric Evidence for Anaerobic Acid Production.* Manometric studies support the analytical findings for anaerobic acid production. The usual Warburg technique was employed; a gas mixture of  $N_2$  and 5%  $CO_2$  was passed through a heated copper coil to give completely anaerobic conditions;  $M/15\ KH_2PO_4$  or Ringer's Ca-free buffers were used. In Fig. 3 the  $Q_{CO_2}^{N_2}$  (phosphate buffer) is 59 for the first

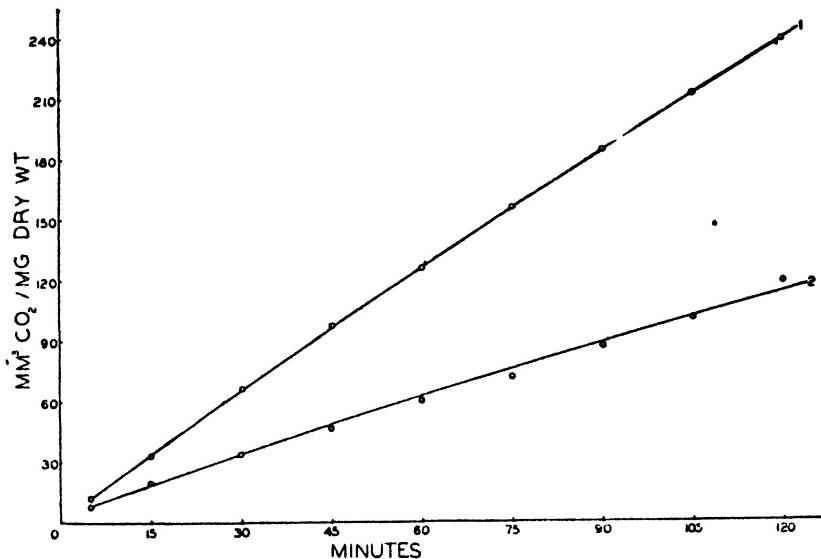


FIG. 3

Anaerobic  $CO_2$  production by *Z. acidifaciens*. (1) bicarbonate buffer, pH 7.9, (fermentation plus acid  $CO_2$ ); (2)  $M/15\ KH_2PO_4$  buffer, pH 4.5 (fermentation  $CO_2$  only). 1.94 mg. yeast dry wt./vessel, 5 mg. glucose/vessel, 1.5 cc. liquid volume/vessel. Temp. 28.00°C., 24 hour yeast culture used,  $N_2$ —5%  $CO_2$  gas phase.

hour and 53 for the second while the  $Q_{acid}^{N_2}$  (total  $CO_2$  released in bicarbonate buffer minus the  $CO_2$  produced in phosphate buffer) is 61 for the first hour and 60 for the second. Thus the volume of  $CO_2$  from fermentation and that from neutralization of the acid formed are roughly equal. The conditions in the various vessels were not the same, however, as both bicarbonate and phosphate buffers were used, giving pH values of 7.9 and 4.5 respectively at the beginning of each run. At the above mentioned first-hour rates, 0.237 mg. of glucose/hour

were fermented to  $\text{CO}_2$ , and 0.164 mg. of acetic acid (assuming for the purposes of this calculation that it was the only acid formed) were neutralized/hour. This represents an enormous amount of acid though the use of different buffers may account for some of the quantity apparently made. The large scale experiments do not result in yields of acid of such an order; conditions in the flasks could not be called absolutely anaerobic however.

### Glycerol Production

Acids being oxidized products, one must look for the reduced counterpart. Since the higher alcohols had been excluded by the results of the Duclaux distillations mentioned previously, it was most logical to search for glycerol.

Erlenmeyer flasks of 250 cc. capacity containing 200 cc. of the basal medium with 10% glucose were inoculated with *Z. acidifaciens* and incubated for 10 days at 30°C. in anaerobic Smillie jars. At the end of 10 days the medium was centrifuged to remove yeast cells and the centrifugate analyzed. 25 cc. samples were treated with 20% copper sulfate and 10% calcium hydroxide to give sugar free filtrates. The filtrate was analyzed for glycerol by the Wood and Werkman modification (1940) of Malaprade's periodate titration. Fifty cc. samples of the centrifugate were titrated hot for total acid, then made strongly alkaline and steam distilled to remove alcohol which was measured by the Gettler and Ereiraich (1931) method of oxidizing to acetic acid. Other samples of the centrifugate were analyzed for glucose, volatile and non-volatile acids as previously described.

TABLE IV  
*Glycerol and Acid Production by Z. Acidifaciens from 10% Glucose-Basal Medium*  
*Flasks incubated anaerobically for 10 days at 30°C.*

Calculation	Glucose Consumed	Products			
		Ethanol	Acetic Acid	Lactic Acid	Glycerol
mM/liter	316	239	45	6	125
per cent of glucose consumed		19	5	1	22
g./100 cc.	5.70	1.10	0.27	0.05	1.25

Under these conditions glycerol production averaged 1.25%, alcohol 1.10%, acetic acid 0.45%, and lactic acid 0.05% (see Table IV). The sugar analyses showed 5.7% consumption; 22% of the glucose consumed was recovered as glycerol, 19% as ethanol, 8% as acetic acid, and 1% as lactic acid; the balance would be accounted for by growth

and  $\text{CO}_2$  production. These data are in fair agreement with the Neuberg and Hirsch (1919) basic fermentation equation:



This equation was deduced from fermentations conducted under alkaline conditions. The possibility of a more general distribution of this type of fermentation as a "natural" one among the so-called acid-producing yeasts, as mentioned in the Introduction, has not apparently been investigated. Certainly, demonstration of an oxidized metabolic product demands consideration of a reduced product.

#### *Respiration of Z. Acidifaciens*

As pointed out in a previous paper (Nickerson and Carroll, 1943) the  $Q_{\text{O}_2}$  of this yeast is affected greatly by the age of the cells making up the suspension. Variation in the  $Q_{\text{O}_2}$  with the concentration of glucose substrate is shown in Fig. 4. Under aerobic conditions a

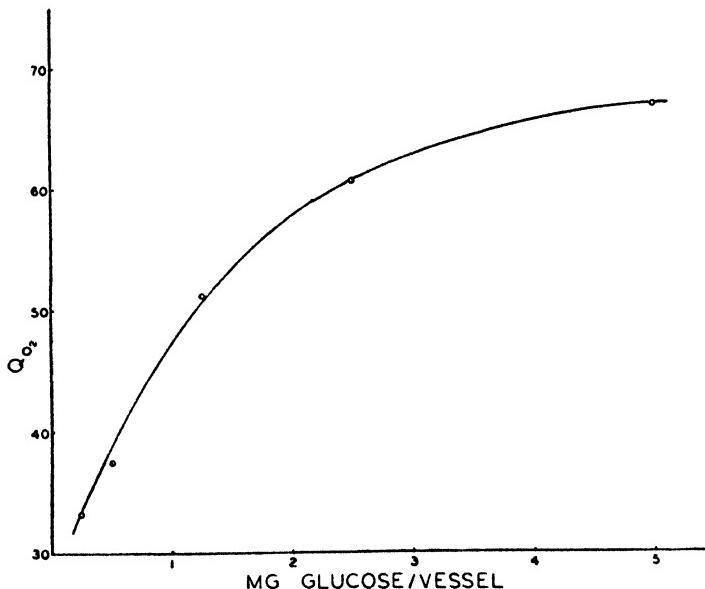


FIG. 4

Effect of substrate concentration on the rate of oxygen consumption by *Z. acidifaciens*. 2.62 mg. yeast dry wt./vessel, gas phase air, 2.5 cc. liquid vol./vessel, 24 hour culture used.

strong fermentation persists (Fig. 5). At the end of 4 hours the quotient  $\text{CO}_2/\text{O}_2$  is 1.55, having fallen from a first hour value of 2.13. Thus, while considerable excess  $\text{CO}_2$  is formed aerobically, its rate of formation decreases with time. The endogenous R.Q. (Fig. 5) is 1.14 at the end of 4 hours.

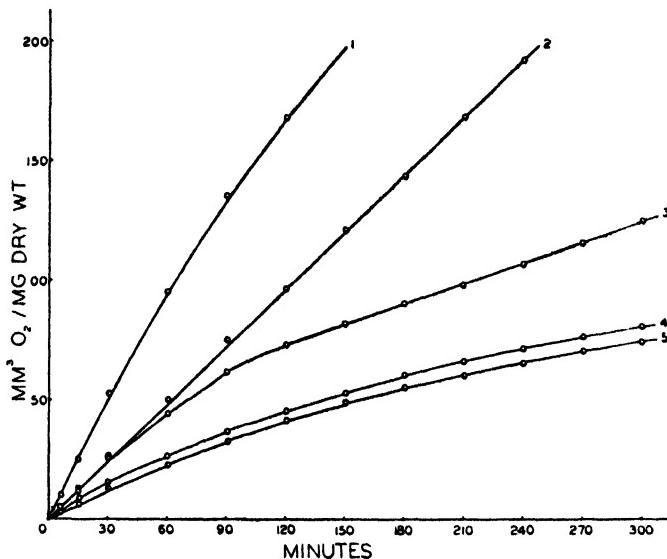


FIG. 5

Oxygen consumption and aerobic  $\text{CO}_2$  production by *Z. acidifaciens*. (1) Total  $\text{CO}_2$  produced—calculated from 2 plus 3, (2) oxygen consumption, (3)  $\text{CO}_2$  produced minus  $\text{O}_2$  consumed, no KOH in inset, (4) endogenous  $\text{CO}_2$  production, (5) endogenous  $\text{O}_2$  consumption. 2.24 mg. yeast dry wt./vessel; 24 hour culture; 5 mg. glucose where substrate was added; temp. 28.00°C.; 2 5 cc. liquid vol./vessel; initial pH 5.0.

The oxidation of glucose by *Z. acidifaciens* has been found (Nickerson and Thimann, 1943) to be unaffected by oxalate, malonate, glutarate, or pimelate in final concentrations of  $10^{-2} M$ ; similarly  $10^{-2} M$  sodium fluoride had no inhibitory effect (see Fig. 6). Oxidation of glucose is completely suppressed by  $4 \times 10^{-3} M$  azide and by the same concentration of cyanide.

No significant oxygen uptake in excess of the endogenous value is exhibited by *Z. acidifaciens* with any of the following substances as the only source of carbon: lactate, glutarate, malonate, pimelate,

malate, fumarate, succinate, citrate, glycollate, or saccharate. These results are in line with those found by Hahn and Haarmann (1928) that baker's yeast does not noticeably oxidize added citrate, succinate, fumarate, or malate. Krebs (1943) has concluded that the tricarboxylic

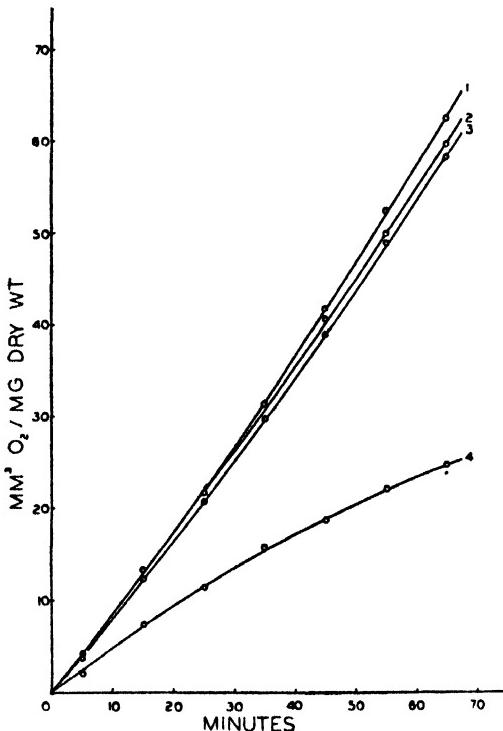


FIG. 6

Oxygen consumption by *Z. acidifaciens* in presence of sodium fluoride. (1)  $5 \times 10^{-4} M$  NaF final concentration, (2) control with glucose, (3)  $10^{-2} M$  NaF, (4) endogenous. 2.34 mg. yeast dry wt./vessel; 5 mg. glucose/vessel with substrate;  $M/15$   $KH_2PO_4$  buffer in all vessels; 2.5 cc. liquid vol./vessel.

acid cycle probably plays little if any part in the oxidation of carbohydrate by the yeasts so far studied; the same appears true of this yeast. However, *Z. acidifaciens* oxidizes glucose, ethanol, and acetate at nearly identical rates as seen in Fig. 7.

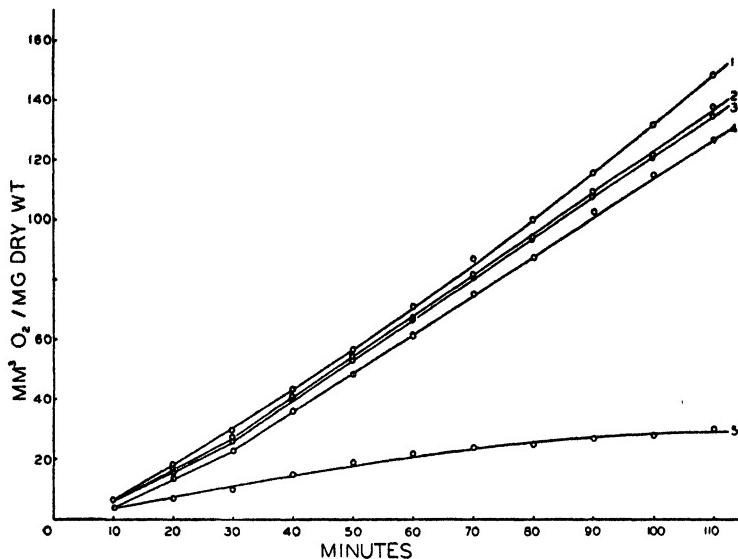


FIG. 7

Oxidation of glucose; ethanol, and acetate by *Z. acidifaciens*. (1) 0.01 M acetate,  $Q_{O_2} = 71$ ; (2) 0.087 M ethanol,  $Q_{O_2} = 67$ ; (3) 0.011 M glucose,  $Q_{O_2} = 66$ , (4) 0.05 M ethanol,  $Q_{O_2} = 61$ ; (5) endogenous. 2.44 mg. yeast dry wt./vessel, 24 hour culture; temp. 28.00°C.; 2.5 cc. liquid vol./vessel.

## DISCUSSION

In the induction period of the hexose fermentation, hexose di-phosphate may be dismuted to the keto-triose, dihydroxyacetone-phosphate, which is reduced to  $\alpha$ -glycerophosphate (glycerol plus  $H_3PO_4$ ), and an aldotriose, glyceraldehydephosphate which is oxidized to 3-phosphoglyceric acid. After acetaldehyde is formed it usually replaces dihydroxyacetonephosphate as a hydrogen acceptor because of the affinity (see Negelein and Brömel, 1939) of the activating protein for acetaldehyde in this DPN hydrogen transfer. No work had been done as yet that throws any light on the actual mechanism whereby acid and glycerin are produced by this yeast but it seems clear that we have here a case of alternate pathways to the "normal" alcoholic fermentation. Many possibilities exist, one or more of which may account for the apparently simultaneous production of the five significant fermentation products: ethanol, carbon dioxide, acetic acid,

lactic acid, and glycerol. A Cannizarro reaction might account for the ethanol and acetate but since much more ethanol than acetate is produced, it would have to be superimposed on the normal alcoholic fermentation. A dismutation of pyruvic acid to acetic and lactic acids has been shown by Barron and Lyman (1939) for some bacteria. And the oxidative decarboxylation of pyruvic acid discovered by Lipmann (1940) leading to acetic acid and  $\text{CO}_2$  permits a hydrogen acceptor which conceivably could be dihydroxyacetonephosphate. It appears in general, however, that cells having a powerful carboxylase system do not oxidize pyruvate.

While conditions in the Smillie jar were quite anaerobic as indicated by a methylene blue inset tube, acid production (and possibly glycerol) was less than that obtained in the Warburg vessels with an oxygen-free  $\text{N}_2$  atmosphere; of course no growth occurred in the vessels. As is well known (Nord, 1940) glycerol has been produced commercially by adding alkali and sodium bisulfite to a well-started alcoholic fermentation; recorded yields average 21% of the sugar fermented. Glycerol production by *Z. acidifaciens* is however a direct, continuous process without benefit of such "steering" substances guiding the fermentation.

Although the mechanics of ethanol and acetate oxidation are still obscure, interest in acetate oxidation has revived recently since Lipmann (1942) isolated acetyl phosphoric acid as the precursor of acetic acid in *Bacterium Delbrückii*. While the chemical schemes of oxidation of acetate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  through such intermediates as glycolic acid and glyoxylic acid have been considered at various times, the oxidation of these substances when used as substrates has never been detected. This yeast is no different in this respect since no oxygen uptake could be observed with glycolate as substrate. One must not rely too much on this type of reasoning for, as Lipmann (1941) has pointed out for acetyl phosphate, and Meyerhof, Ohlmeyer, and Möhle (1938) for hexose diphosphate, the relations between oxidation, reduction, and phosphorylation may be so delicately balanced that even a known intermediate may not react unless all conditions are appropriate. Kleinzeller (1943) found acetate oxidation by slices from the guinea pig kidney cortex to be inhibited by malonate, and of many substances tried, only those of the tricarboxylic acid cycle could be oxidized at rates similar to that found for acetate. It is doubtful that succinic dehydrogenase plays any part here in the

oxidation of acetate since this yeast shows no effect of the presence of  $10^{-2} M$  malonate, a specific inhibitor, on the oxidation of glucose; further, the closely parallel high rates of oxygen uptake with glucose, ethanol and acetate suggest the possibility that similar oxidation pathways are involved.

The authors are very grateful to Dr. Fritz Lipmann for most helpful discussion and for reading the manuscript.

#### SUMMARY

A study has been made of the aerobic and anaerobic glucose metabolism of *Zygosaccharomyces acidifaciens*. Anaerobically, acetic acid, ethanol, lactic acid,  $\text{CO}_2$  and glycerol are produced.

Acid production is greater in a medium with an initially acid pH, and increases up to 0.088  $N$  with increasing sugar concentrations up to 30% glucose. The existence of a normally occurring Neuberg type III fermentation is here demonstrated in an acid medium without addition of sulfite. Attention is drawn to the relation between this type of fermentation and the so-called "acid producing" yeasts.

Of the acid produced anaerobically, 70% is volatile acid consisting of acetic only, and of the non-volatile acid 75% is lactic acid; succinic acid was found in traces. Aerobic acid production has not been observed but this may be a result of its rapid oxidation. Measured manometrically under anaerobic conditions as much  $\text{CO}_2$  is released from neutralization of acid in a bicarbonate buffer at pH 7.9 as is produced from fermentation in a phosphate buffer at pH 4.5.

The reduced metabolic products are ethanol and glycerol. From a 10% glucose solution anaerobically, 1.10% ethanol and 1.25% glycerol are produced, accounting respectively for 19% and 22% of the glucose fermented. Possible mechanisms of production for the metabolic products are discussed.

A strong fermentation persists with *Z. acidifaciens* under aerobic conditions. It oxidizes glucose, ethanol, and acetate at nearly equal rates. Its respiration with a glucose substrate is completely inhibited by cyanide and by azide but untouched by  $10^{-2} M$  malonate or fluoride. Mechanisms of oxidation of acetate are discussed.

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## Book Reviews

**Optical Properties of Organic Compounds.** By ALEXANDER N. WINCHELL, Professor Emeritus of Petrology and Mineralogy in the University of Wisconsin. The University of Wisconsin Press, Madison, Wisconsin, 1943. xiii + 342 pp. Price \$5.00.

This is a reference work rather than a book for reading. There are listed from the literature and from hitherto unpublished data that were furnished the author by many colleagues the optical properties of some thirteen hundred crystalline organic compounds, with references to the original sources from which the data have been collected or received. The arrangement of this large list follows the Beilstein system and there is also a comprehensive name index. The author has emphasized the usefulness of optical data, particularly the indices of refraction, for the identification of crystalline organic compounds. Quoting from the introduction, "this volume includes, so far as they are known, all organic compounds whose indices of refraction have been measured." Axial ratios and other crystallographic data have also been included whenever possible. Systematic charts for aid in the identification of a sample of an organic compound from the easily made measurements of its refractive indices are presented. In collecting the measurements from some four hundred original references the compiler must depend upon the original observer for names and formulas in addition to the numerical data; some of these names and formulas that have been carried through from the original articles are now out of date but this will not mislead the specialist, who can readily consult the stated original reference. The author is to be thanked for his arduous labor in preparing this valuable volume under the auspices of the Wisconsin Alumni Research Foundation and with the editorial assistance of Drs. E. Leon Foreman, Minnie Meyers and Ivan Wolff and final editing by Dr. Leonard T. Capell, associate editor of *Chemical Abstracts*.

CLAUDE S. HUDSON, Bethesda, Md.

**The Amino Acid Composition of Proteins and Foods. Analytical Methods and Results.** By RICHARD J. BLOCK, Ph.D., and DIANA BOLLING, B.S. Charles C. Thomas, Springfield, Ill., 1945. 396 pages. Price \$6.50.

The study of protein chemistry has been revived during the last decade. Analytical methods have attained increasing importance especially in regard to practical problems of nutrition and theoretical insight into the processes of metabolism. Block and Bolling have, on their part, contributed to the advancement of the analytical methods involved. The present book contains much valuable data, and, on close inspection, it appears that no essential facts have been omitted. Merely enumerating the details of methods would not serve to indicate the value of the book.

Chapter X gives useful summary tables showing the content of amino acids in numerous animal and plant proteins, including hormones and enzymes, albuminoids, keratins and seed globulins. Furthermore, there are data on grasses, leaves, yeasts and viruses, as well as meat scraps, fish meals and other protein materials.

The authors have endeavored to present a picture of the historical development in the field. In doing so, they should have mentioned the pioneer work of Effront, Loewi and Abderhalden on nutrition with amino acid-mixtures, as well as the important findings on mannose (S. Fraenkel) and galactose (Sørensen) in relation to proteins. The selective adsorption of amino compounds had been applied by L. Hermann (1881), Waldott (1913) and Gordin-Kaplan (1914), many years prior to Whitehorn. E. Fischer, in 1901, was probably the first to use copper salts for the separation of certain amino acid fractions (valine and leucine). Difficulties are encountered in Kjeldahl nitrogen determination, not only with lysine and histidine, but also with tryptophane (Liebermann, 1909). On page 221, seventh line from the bottom, read "hydroxy acids," since, on deamination amino acids do not form hydroxy amino acids. The factor 0.052, ascertained for the calculation of acetone from the weight of the acetone-mercury sulphate precipitate by C. Oppenheimer, is preferred to the factor 0.05, based on this writer's own experience. The isotope dilution method is mentioned only briefly; but the importance and exactness of this method deserve a more detailed description. These remarks may be taken into consideration in planning a second edition which, doubtless, will soon be necessary.

All in all, this book is a definite contribution to the enrichment of the special literature in this field.

C. NEUBERG, New York, N. Y.

**Root Disease Fungi.** By S. D. GARRETT, Mycologist, Rothamsted Experiment Station. The Chronica Botanica Company, Waltham, Massachusetts; G. E. Stechert and Company, New York, N. Y., 1944. 177 pp., illus. Price \$4.50.

The author states that he has written this book to emphasize the relationship of the root-infecting fungi and its habitat, the soil. He points out that over the globe, especially in cultivated soils, the soil environment of micro-organisms and plants varies less widely than the above-ground environment. Control of root diseases, therefore, is essentially the same whether encountered in the tropics or temperate regions and methods of control vary more with the type of cultivation.

The plant pathologists still have much to learn about soil biological factors in the control or regulation of soil pathogens in combating various soil-borne diseases. Undoubtedly, the surface of this subject has just been scratched and we should look forward towards newer developments in biological relationships which will aid in the control of plant diseases.

The publication of this excellent book is timely and brings together and elucidates the more important published data on the subject. The method of presentation and arrangement of the subject matter are logical and clear-cut. It certainly brings together a vast fund of information in a manner which should stimulate continued investigations towards a promising goal of control for many of our complex soil plant-disease problems.

In the introduction is given a short history of soil-disease investigations. It was pointed out that the soil environment exercised a profound effect upon the development of most soil-borne diseases; for example, the relation of soil temperature to plant disease. The importance of the microbiological factors in the soil environment of the root-infecting fungi was shown to be important, especially in the curtailment

of the saprophytic existence of root-infecting fungi. Such factors as antagonism and even actual parasitism of one micro-organism upon another are other examples. These discoveries led to application of the same for root-disease control. In Chapters 2 through 8, the author discusses the behavior of the root-infecting fungi and demonstrates certain trends in the subterranean evolution of the parasitic habit. A splendid job of bringing together and elaborating upon investigations of the following subjects is presented: Parasitic specialization and activity of root-infecting fungi; influence of soil temperature, soil moisture, soil organic content, and concentration of plant nutrients upon parasitic activity; and the saprophytic activity and dormancy of the root-infecting fungi. The remaining chapters are devoted to principles of root-disease control under different types of cultivation. Here is presented a detailed account of control of root disease in field crops by plant sanitation, disease control under the growing crop, in virgin soils, in mature plantations and on replanted areas, and for special problems. The final chapter is devoted to control of root disease in glass-house crops. An excellent bibliography and general and authors' index are included.

This volume is a real contribution to a much-neglected field. Investigators interested in these problems will obtain a well-organized and clearly stated picture based upon the more important investigations conducted with root-disease fungi.

OTTO A. REINKING, Geneva, N. Y.

**An Introduction to Pollen Analysis.** By G. ERDTMAN, Ph.D., Västerås, Sweden. The Chronica Botanica Co., Waltham, Mass.; G. E. Stechert and Co., New York, N. Y., 1943. xv + 239 pp. Illustrated. Price \$5.00.

Twelfth in the New Series of Plant Science Books from this press, this volume concerns itself with a field that has been largely developed within the last thirty years. The work of an outstanding investigator in pollen analysis, it represents a summary of the contributions already made and a view of possible important future progress. The bibliography is comprehensive and the subject matter touches many fields of science so that workers in various phases will profit by this thorough organization of the results of studies available to the author in Sweden during the early war years; a supplementary list of publications has been added through the offices of the editors by two American investigators.

For the chemist, possibly the greatest interest will center in the chapter on the chemistry of peat and those on the preparation of pollen and fossil pollen-bearing material. A consideration of the preservation of pollen on the basis of chemistry can be related to the techniques used for fresh and fossil pollen. Practical methods are clearly presented.

Six chapters, comprising over a hundred pages and carrying many illustrations of great value in identifying pollen and spores, are grouped under Morphology. These as well as the subject of Tertiary deposits appeal especially to the botanist. Studies of Quaternary deposits are listed by countries in a geographical survey involving about 120 titles.

Attention is also given to the methods of recording and graphing pollen analyses with great emphasis on the complex symbols developed in Europe. Not the least interesting material considers the output and dissemination of pollen, what may happen to it upon settling upon a surface, and the history of such pollen as settled

long enough ago to be now a part of the flora in peat. And to round out the analysis, there is a final brief chapter on pollen in honey and drugs.

With full appreciation of the excellent volume produced in spite of difficulties, one nevertheless hopes that subsequent editions will avoid the lengthy direct quotations from papers in German, French, and English, reserving direct quotation for controversial points. Eventually also it is to be hoped that a simpler direct terminology may displace the excessively developed and awkward terminology, often characteristic of the early phases of a subject. A very useful volume will then be enhanced in readability for the wide range of ecologists, foresters, geologists, chemists, climatologists, and general botanists aside from the specialist in pollen analysis.

NORMA E. PFEIFFER, Yonkers, N. Y.

# The Synthesis of Para-aminobenzoic Acid by Yeast \*

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Received April 16, 1945

## INTRODUCTION

Many investigators have selected yeast as the most convenient source of sulfonamide inhibitor or *p*-aminobenzoic acid (P.A.B.). In his original identification of the sulfonamide inhibitor as P.A.B., Woods (1) took bakers' yeast as his source material. Early in our work on the isolation of the growth substances required by *Clostridium acetobutylicum*, yeast and yeast extracts were also found to be excellent sources of the factor we had termed BY (2). This factor was subsequently identified with P.A.B. (3), which has been isolated from yeast by several investigators (4, 5, 6).

Landy and Dicken (7) have reported yeast capable of synthesizing considerable amounts of a sulfonamide inhibitor during growth. Lewis, *et al.* (8) also studied vitamin production by torula yeast and observed an average synthesis of 56γ of P.A.B. per g. of dry yeast. We had previously observed the synthesis of large amounts of P.A.B. by bakers' yeast and have studied this in detail, under both commercial and laboratory conditions, in both natural and synthetic media, the results being reported in this paper. Optimum methods of extraction of the P.A.B. from yeast have also been determined. The P.A.B. values were obtained by assay with *Cl. acetobutylicum* (9) or with *Acetobacter suboxydans* (10). Most of the samples were run against both organisms as a check on the agreement and specificity of the two methods.

## EXPERIMENTAL

### Determination of P.A.B.

The assays with *Cl. acetobutylicum* S9 were carried out according to the technique of Lampen and Peterson (9). The medium of Landy and Dicken (10) was chosen

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station and supported in part by a grant from the Wisconsin Alumni Research Foundation.

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for the determinations by *Acet. suboxydans*, A.T.C.C. 621. However, the purified casein hydrolyzate was that prepared for use with *Cl. acetobutylicum*. We were unable to demonstrate a stimulation of *Acet. suboxydans* by purines (11) under our conditions. Hence, these substances were not included in the basal medium. A standard Difco yeast extract sample was run with each assay as an additional check of the method. All other details of the procedures were identical with the published methods. The criterion of growth was turbidity read in the Evelyn Colorimeter with readings at 24 hrs. for *Cl. acetobutylicum*, and at 48 hrs. for *Acet. suboxydans*. With the latter organism, the culture was homogenized before being read. Unless this was done erratic results were obtained with both the standard and unknown samples.

Blanchard (6) reported an increase after autolysis of yeast both in the P.A.B. which could be isolated and in the total diazotizable amines present. Kuhn and Schwarz (5) reported that they were able to obtain complete extraction of P.A.B. from dried yeast with boiling water, but that they obtained only 15-25% of the P.A.B. from fresh yeast by this procedure. Hydrolysis with 2 N H<sub>2</sub>SO<sub>4</sub> caused no destruction. Auhagen (12), likewise, obtained higher amounts of acetone-extractable P.A.B. from yeast following autolysis. Using the Neurospora method, Thompson, *et al.* (13) reported increased P.A.B. values from moist yeast on acid autoclaving (6 N H<sub>2</sub>SO<sub>4</sub>, 1 hr.) over those obtained from the water extract. However, the increase was not great. Water extraction gave 3.6γ per g. of moist tissue as compared to 4.0γ per g. after acid hydrolysis. Lewis (14), with *L. arabinosus* as test organism, found up to 40% of the P.A.B. in yeast bound. This combined form was released by autoclaving with 1 N NaOH for 30 min. Large increases in the P.A.B. content of liver, peptone, urine, etc., have been observed during acid or alkaline hydrolysis (9). P.A.B. exists in these materials in a combined form which is unavailable to *Cl. acetobutylicum*. Much of this bound P.A.B. is water-soluble. Alkaline hydrolysis gave the best liberation, autoclaving in 5 N NaOH at 75-80 lbs. for 1 hour generally giving optimum results.

In Table I the P.A.B. values obtained on yeast by different extraction procedures and by the two microbiological assay methods are given. Practically all of the P.A.B. may be extracted with water. Treatment with acid, either HCl or H<sub>2</sub>SO<sub>4</sub>, caused losses of up to 50%. Autolysis under our conditions caused no increase in potency. This is contrary to the observations of Blanchard (6) and Auhagen (12). In experiments with alkaline hydrolysis some increases over the water-soluble P.A.B. have been noted. These have usually been 5-15% of the total P.A.B. One experiment with bakers' yeast, Brand B, gave 43% bound P.A.B. It is evident that while this combined form does occur in yeast, its percentage is much less than in liver, peptone, or urine. Lewis, *et al.* (8) were unable to demonstrate any alkaline-hydrolyzable P.A.B. in torula yeast. In our study of P.A.B. synthesis reported later, we found that water extraction generally gave values as high as or higher than those obtained from the alkali treatment.

TABLE I  
*Microbiological Assays of P.A.B. Samples*

<i>Sample*</i>	By <i>Cl. acetobutylicum</i> γ/g. dry matter	By <i>A. suboxydans</i> γ/g. dry matter
1. Foil Yeast, Brand A		
H <sub>2</sub> O, 1 hr.	28	24
1 N H <sub>2</sub> SO <sub>4</sub> , 1 hr.	13.5	
2 N H <sub>2</sub> SO <sub>4</sub> , 1 hr.	17	
2. Foil Yeast, fresh, Brand B		
H <sub>2</sub> O, 1 hr.	39	34
3. Bakers' Yeast, fresh, Brand B		
H <sub>2</sub> O, 1 hr.	50	
5 N NaOH, 1 hr.†	88	
2 N HCl, 1 hr.	42	
Autolyzed, pH 4.5, 28 hrs.	43‡	
Autolyzed, pH 7, 28 hrs.	50‡	
4. Bakers' Yeast, dried, Brand B		
H <sub>2</sub> O, 1 hr.	32	35
2 N NaOH, 6 hrs.	38	39
2 N H <sub>2</sub> SO <sub>4</sub> , 1 hr.	25	19
2 N HCl, 1 hr.	24	23
5. Bakers' Yeast, enriched, fresh, Brand B		
H <sub>2</sub> O, 1 hr.	61	62
6. Bakers' Yeast, fresh, Brand C		
H <sub>2</sub> O, 1 hr.		45
5 N NaOH, 7 hrs.		54
7. Bakers' Yeast, dried, Brand D		
H <sub>2</sub> O, 1 hr.	135	128
8. Bakers' Yeast, fresh, Brand E		
H <sub>2</sub> O, 1 hr.		167
5 N NaOH, 7 hrs.		175
9. Yeast Extract (Difco)	80	86
10. Liver A		
H <sub>2</sub> O, 1 hr.	1.7	1.4
2 N HCl, 1 hr.	4.0	5.6
5 N NaOH, 5 hrs.	11.8	11.4

\* For all hydrolyzates, 1 g. of the material was autoclaved with 50 ml. of the appropriate reagent at 15 lbs. pressure.

† Autoclaved at 75 lbs. pressure.

‡ Values at 72 hrs. identical within experimental error.

For this reason, the data presented are for water extracts of the yeast or for the unhydrolyzed fermentation medium, unless otherwise stated.

The data of Table I illustrate an excellent agreement between the assays by the two organisms. Three preparations from Liver A (9) have been included to broaden this comparison. The data were analyzed statistically by the method of paired differences (15). The value of *t* found (0.56) is not significant for eleven degrees of freedom. Hence we are justified in concluding that the methods do not differ significantly.

Evidently the "bound P.A.B." is unavailable to either organism. After acid or alkali treatment of yeast or liver, approximately the same amounts of P.A.B. are available to both organisms. The specificity of each organism has been investigated (10, 16) in some detail. Our findings are additional evidence that the active entity is the same for both.

Large variations were noted in the P.A.B. content of different samples of the same brand of yeast, *e.g.*, B. The samples were obtained at different times so they do not represent the same batch of yeast. At the time the samples were analyzed our primary interest was centered on the reliability of the two methods and their agreement with one another. Later a number of samples of commercial yeast were analyzed by the *Acet. suboxydans* method with results as follows ( $\gamma$  per gram of dry yeast): Brand A, 1 sample of bakers' yeast, 22; Brand B, 4 samples of foil yeast, 31 to 53; Brand B, 3 samples of bakers' yeast, 59 to 65.

#### *Synthesis of P.A.B. During Commercial Yeast Production*

In Table II a typical run under commercial conditions for yeast production is outlined. The difference in volume between the sum of the feed wort and yeast inoculum (70.8 l.) and the fermented wort and yeast (295 l.) is due to water added in the course of the fermentation. The P.A.B. rose from 93.7 mg. in the original materials to 1,829 mg. in the fermented material. This is approximately a twenty-fold increase. Thirty per cent of the P.A.B. in the fermented material and in the yeast inoculum was found in an alkali-hydrolyzable form, and 70% of that in the feed wort was bound. The total P.A.B. values are listed in Table II. Seventy-nine per cent of the total P.A.B. in the fermented mixture was present in the medium, and only 21% in the cells. Lewis, *et al.* (8) found 55% of the total P.A.B. to be present in the medium

TABLE II

*Synthesis of p-Aminobenzoic Acid by Yeast Under Commercial Conditions\**

## Feed Wort

Total volume, liter.....	60.6
P.A.B. concentration, mg./l.....	0.25
Total P.A.B. in wort, mg.....	15.2

## Yeast Inoculum

Total volume, liter .....	10.2
P.A.B. concentration, mg./l.....	7.7
Total P.A.B. in inoculum, mg.....	78.5

## Fermented Wort and Yeast

Total volume, liter .....	295
P.A.B. concentration, mg./l.....	6.2
Total P.A.B. in fermented material, mg. ....	1,829

## Yeast

Total solids, kg. ....	10
P.A.B. concentration, mg./kg. ....	38.5
Total P.A.B. in yeast, mg. ....	385

## Vitamin Balance

Total P.A.B. in feed wort and yeast inoculum, mg. ....	93.7
P.A.B. in cell-free fermented wort, mg. ....	1,444
P.A.B. synthesized, mg. ....	1,735
P.A.B. in cell-free fermented wort, % of total P.A.B. present....	79

\* All samples autoclaved with 5 N NaOH for 6 hrs. before assay. All assays performed with *Cl. acelobutylicum* S9.

after the growth of torula yeast. Another sample of fermented wort assayed 3.0 mg. of P.A.B. per liter without hydrolysis and 4.1 mg. per liter after hydrolysis with 5 N NaOH at 75-80 lbs. pressure for 1 hour.

In commercial manufacture of yeast the final step in the separation of the yeast is to run a thick suspension of cells through filter presses. The filtrate or pressings from a commercial batch of yeast contained 1.4 mg. of P.A.B. per liter as assayed without hydrolysis. This material had 1.5 g. of solids per liter, i.e., a P.A.B. concentration of 930γ of P.A.B. per gram of solids, which is about twenty times higher than the yeast itself. It is evident that P.A.B. passes readily out of the yeast cell into the medium.

*Experimental Yeasts*

More complete data were obtained on yeast grown in the laboratory where all quantities and conditions were known more accurately. In

Table III the P.A.B. synthesis on six different molasses media is given. These media contained varying amounts of beet, invert, and refiners' blackstrap molasses, with 1.0 g. of monobasic ammonium phosphate, and 1.0 g. of ammonium sulfate per 100 cc. In order to compensate for

TABLE III  
*Synthesis of P.A.B. by Yeast on Molasses Media*

Run*	Dry Cells, g./l. (a)	P A B Content†			P A B Increase, γ/l.‡ (a × d) + c - b	% Total P A B in Medium
		Of Unfermented Medium, γ/l. (b)	Of Fermented Cell-free Medium, γ/l. (c)	Of Cells, γ/g. (d)		
I	7.90	16	1,120	11.5	1,200	92
II	7.69	15	1,120	12.0	1,200	92
III	7.60	16.5	780	16.5	890	86
IV	7.33	17.5	1,270	11.4	1,340	95
V	4.65	29	910	9.1	920	96
VI	6.69	16.5	790	11.2	850	91

\* Proportions of molasses (g. per 100 cc.)

- I. Beet, 6, refiners' blackstrap 2
- II. Beet 4.8, invert 2.4, refiners' blackstrap 0.8
- III. Beet 4.8, invert 1.9, refiners' blackstrap 1.3
- IV. Beet 4.8, refiners' blackstrap 3.2
- V. Beet 8
- VI. Refiners' blackstrap 8

† All assays performed with *Cl. acetobutylicum* S9.

‡ Calculated values rounded off to nearest 10.

the lower nitrogen content of the molasses in Runs V and VI, the ammonium phosphate and ammonium sulfate additions were increased to 1.5 g. per 100 cc. All media were initially low in P.A.B. (15–29γ per liter). The P.A.B. synthesized was between 850 and 1,200γ per liter. (In making these calculations, the P.A.B. in the inoculum was neglected as it amounted to less than 5% of the total.) These are thirty-two to eighty-fold increases over the original P.A.B. present. Although the yield of cells was low on the 100% beet molasses medium, the P.A.B. synthesis was not altered markedly by the various combinations of molasses. The P.A.B. content of the yeast grown on these molasses media was relatively low compared to that of yeast later grown on synthetic media and to commercial yeasts.

To supplement the observations on natural materials, a series of runs were made on synthetic media (Table IV). The P.A.B. formation was approximately the same on these synthetic media as on the molasses-salts media, *i.e.*, 1 mg. per liter (Table III). The yield of cells was, however, appreciably lower. In parallel runs on the synthetic medium of Van Lanen, *et al.* (18), a significantly greater P.A.B. formation was obtained with higher levels of the essential factors for yeast.

TABLE IV  
*Synthesis of P.A.B. by Yeast on Synthetic Media*

Medium*	Dry Cells, g./l.	P.A.B. Content†		P.A.B. Synthesized, γ/l.			% Total P.A.B. in Medium
		Of Cells, γ/g.	Of Fermented Cell-free Medium, γ/l.	Cells	Cell-free Medium	Total	
I	2.45	7.8	1,240	20	1,240	1,260	98
II	3.35	18.4	1,000	62	1,000	1,062	94
III	5.24	34.3	1,200	180	1,200	1,380	87
IV	6.60	94.5	2,600	624	2,600	3,224	81

- \* I. Synthetic medium of Williams, *et al.* (17) excepting pantothenic acid and β-alanine, which were omitted.
- II. As I with 1000γ of β-alanine and 2000γ of α, γ-dihydroxy-β, β-dimethyl-γ-butyrolactone per liter.
- III. Synthetic medium of Van Lanen, *et al.* (18) with 100γ per liter of thiamine, pyridoxin, and calcium pantothenate, 1.0γ per liter of biotin, 10 mg. per liter of inositol, and 2 g. per liter of casein hydrolyzate.
- IV. As III, with 1.0 mg. per liter of thiamine and calcium pantothenate, 200γ per liter of pyridoxin, 7.0γ per liter of biotin, and 100 mg. per liter of inositol.

† All assays performed with *Cl. acetobutylicum* S9.

The yields on Medium IV approximate those obtained commercially. In contrast to our results in the commercial runs on complex media, the P.A.B. produced on the synthetic media is in a form available to the assay organism; in fact, alkaline hydrolysis caused 25–40% losses.

#### DISCUSSION

While, under our test conditions, autolysis or enzyme action did not bring about liberation of extra P.A.B. from yeast, alkaline hydrolysis did demonstrate the existence of small amounts of bound P.A.B.

A maximum of 40% of the P.A.B. was in this form in the various yeasts tested. This contrasts with liver and peptone where 80-95% of the P.A.B. was found in conjugated form. The P.A.B. of yeast was relatively labile to acid hydrolysis. This agrees with our previous findings (9) that P.A.B. is generally more stable to alkali than to acid.

Our results show a close agreement between P.A.B. values obtained on yeast and liver by assay with *Cl. acetobutylicum* and by *Acet. suboxydans*. The active entity appears to be the same for both organisms.

It is interesting that the P.A.B. formed during yeast growth on synthetic media was the free acid, while on natural media a considerable portion of the P.A.B. was bound. The magnitude of the synthesis (1-6 mg. per liter) is surprising, as is also the passage of the compound into the surrounding medium and the slight retention in the cell. Seventy-nine per cent of the total P.A.B. was excreted into the medium in the commercial run, 86-96% in the molasses series, and 81-98% in the experiments with synthetic media. However, the greater the amount of P.A.B. synthesized, the larger was the fraction remaining in the yeast cells. This is illustrated in Table IV. Also, in the commercial fermentation, where the greatest synthesis occurred, 21% of the P.A.B. remained in the cells.

The excretion by yeast of the greater part of its P.A.B. production into the medium is in direct contrast to the avidity with which it absorbs thiamine from the medium. Van Lanen, *et al.* (18) reported that practically all of the thiamine present was absorbed until a level of 3 mg. per g. of yeast was attained. Lewis, *et al.* (8) found that *Torulopsis utilis* excreted about 40% of its synthesized riboflavin and pantothenic acid into the medium. Little absorption or excretion occurred with thiamine, nicotinic acid, biotin, or pyridoxin.

The P.A.B. content of the yeast from the molasses media and from Media I and II of Table IV is well below that found in the various commercial samples given in Table I. Only in the commercial run and in the synthetic Media III and IV were the concentrations in the same range as those of the market samples. The addition of large amounts of five yeast growth factors in Medium IV increased both total synthesis and the P.A.B. content of the cells markedly.

It is possible that the synthesis of P.A.B. observed in the natural media consisted simply of the conversion to P.A.B. of some "precursor" present before inoculation. However, considerable synthesis did occur on media containing no organic constituents except sucrose, aspartic

acid, and vitamins (Media I and II, Table IV). Also, an increase in the vitamin supplements stimulated P.A.B. synthesis markedly. The yeast is certainly able to fabricate the P.A.B. molecule in large amounts from simple substances. The existence of a "precursor" in wort or molasses cannot, however, be excluded on the basis of the present data.

### SUMMARY

The P.A.B. content of various yeast samples was determined with *Clostridium acetobutylicum* and *Acetobacter suboxydans*. Assays by the two organisms were in good agreement.

Most of the P.A.B. in yeast occurs free, *i.e.*, in a soluble form available to the assay organisms. Destruction occurred during acid hydrolysis. Autolysis failed to release any P.A.B. Autoclaving with 5 N NaOH gave slightly increased values over water extraction.

P.A.B. was synthesized in large amounts during the growth of yeast. Twenty- to eighty-fold increases occurred both under commercial conditions and on synthetic media. One to six mg. of P.A.B. were produced per liter. Eighty per cent or more of this was found in the medium, and only a small percentage remained in the yeast cells.

### ACKNOWLEDGMENT

The authors are indebted to Helen Massock and J. E. Oyaas for growing the yeasts and for data on yields reported in Tables III and IV. They are also obliged to Dr. C. H. Keipper of the Red Star Yeast and Products Company, Milwaukee, Wisconsin, for the samples of wort and yeast analyzed in Table III, and for data on the interrelation of the two materials.

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# Studies on Vitamin "Bc" Produced by Microorganisms \*

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Received March 31, 1945

## INTRODUCTION

Numerous investigations of the "norite eluate" obtained from extracts of natural materials, such as liver, leaves and yeast, have demonstrated the existence of potent vitamin-like substances which are essential for the growth of some bacteria and active in preventing nutritional cytopenia in certain birds and mammals. Highly purified preparations of the eluate materials obtained from various natural sources have been found to possess great activity for *Lactobacillus casei*, or for *Streptococcus lactis*, or in some instances for both of these bacteria. A review of this field having appeared recently (1), our discussion of the literature will be restricted to the particular contributions which seem to be relevant to problems concerned with the production by microorganisms of a factor (or factors) which stimulates the growth of *L. casei* cultivated in a special type of medium.

The activity of crude natural materials which function in much the same way as does crystalline vitamin Bc (2) will be referred to in this paper as "vitamin Bc" or "*Lactobacillus casei* factor". It should be realized that the methods used in our assays provide analytical data representing the total activity of the special substances, such as vitamin Bc and thymine,† which promote growth of *L. casei* under specified conditions.

Although certain natural materials, such as yeasts and yeast extracts, show vitamin Bc activity when fed to anemic chicks or nutritionally cytopenic monkeys, these materials supplied directly to *Streptococcus lactis* and *Lactobacillus casei* appear to have relatively

\* This work was supported by grants from Parke, Davis and Company and the Williams-Waterman Fund of Research Corporation.

† Krueger, K., and Peterson, W. H., *J. Biol. Chem.* **158**, 145-156 (1945).

much lower potency for promoting growth of the bacteria. The successful isolation of crystalline vitamin Bc from yeast which had been subjected to preliminary enzymatic digestion (3), together with other evidence obtained in various experiments with enzyme preparations (4) has led to the conclusion that vitamin Bc in yeast, and perhaps also in other materials, exists chiefly in the form of a simple non-protein conjugate. Substantial increases of a factor (or factors) promoting growth of certain lactic acid bacteria have been observed during incubation of various reaction mixtures, such as chicken liver and yeast, or rat liver and yeast (5, 6), rat liver and xanthopterin, or urine, or extract of grass (7, 8), and living cultures of *S. lactis* R. in the presence of a special factor (9). It now appears that, from a nutritional standpoint, assays of vitamin Bc may have greater significance if the determinations are performed with methods designed to test the total (free + conjugated) rather than only the free Bc. Recognition of the need for preliminary treatment of samples is evidenced in the recent use of enzymatic digestion (6, 10) and acid hydrolysis (11) prior to assay. Comparison of various methods employed for growing the assay bacteria (12) indicates the importance of employing an adequate basal medium.

The purpose of this paper is to describe a simple method of preparing samples for vitamin Bc assay with *L. casei*, and to report some observations on the vitamin Bc produced by certain bacteria, yeasts and molds.

## EXPERIMENTAL

### *Method of Vitamin Bc Assay*

The titrimetric determination of vitamin Bc activity by means of *Lactobacillus casei* depends upon the quantitative production of lactic acid during growth of the bacteria in proportion to the vitamin available in a suitable culture medium. The assay method is essentially a simple titration with 0.1 *N* NaOH to neutralize the acid produced during a standard growth period of 72 hours. The basal medium employed for the assay organism in this study has essentially the composition recommended in a recent paper from the University of Texas (13). The ingredients for double strength medium are given in Table I.

The general techniques employed in performing the analyses are those now in common use for microbiological assay (12, 14). The inoculum is prepared from a 24-hour culture of *L. casei* grown in 10 ml. of yeast-dextrose-broth. The bacteria are separated from the medium by centrifugation, and the organisms are resuspended in 10 ml. of sterile physiological saline and centrifuged a second time. The supernatant solution is decanted and the bacteria are again suspended in 10 ml. of saline. One

ml. of this suspension is pipetted aseptically into 20 ml. of sterile saline solution, and the suspension is mixed by shaking. One small drop of this inoculum is transferred with a fine-tipped sterile pipette into each assay tube. Using this method the inoculum is practically free from Bc and satisfactory blank titrations are obtained.

TABLE I

*Stock Basal Solution for the Assay of "L. casei Factor"*

Casein amino acids	10.0 g.
Glucose	20.0 g.
Sodium acetate	12.0 g.
Asparagine	0.5 g.
Tryptophan	0.2 g.
Cystine	0.2 g.
Salt solution A*	10.0 ml.
Salt solution B*	10.0 ml.
Glutamine	10.0 mg.
Guanine	10.0 mg.
Adenine	10.0 mg.
Xanthine	10.0 mg.
Uracil	10.0 mg.
Thiamine	200.0 γ
Biotin	1.0 γ
Riboflavin	400.0 γ
Calcium pantothenate	400.0 γ
Nicotinic acid	400.0 γ
Pyridoxin	1200.0 γ
p-Aminobenzoic acid	400.0 γ
Water to make	1000.0 ml. of solution

\* The composition of salt solution A is as follows:

$K_2HPO_4$ , 10.0 g.;  $KH_2PO_4$ , 10.0 g.; water to make 100 ml.

The composition of salt solution B is as follows:

$MgSO_4 \cdot 7H_2O$ , 4.0 g.;  $NaCl$ , 0.2 g.;  $FeSO_4 \cdot 7H_2O$ , 0.2 g.;  
 $MnSO_4 \cdot 4H_2O$ , 0.2 g., water to make 100 ml.

The graph in Fig. 1 shows a typical dosage-response curve after incubation for 72 hours at 37°C. Preliminary tests showed that the acid production curve levels off at the end of a 72 hour period of incubation, provided glutamine is present in the medium. Maximum acid production in this medium without glutamine was obtained after 120 hours. Each level of response to the vitamin † is represented in the figure by titrations of triplicate 10 ml. cultures. A typical set

† Crystalline Bc supplied by Parke, Davis & Co.

of values obtained for an enzyme-digested sample of yeast is shown beside the curve.

It is apparent that good agreement is obtainable among the individual titrimetric determinations made on aliquots of the sample and corresponding in vitamin content to different levels on the standard curve. Addition of bacteria-fermented yeast extract to the assay tubes, in amounts sufficient to supply 80 times the dose of Bc required

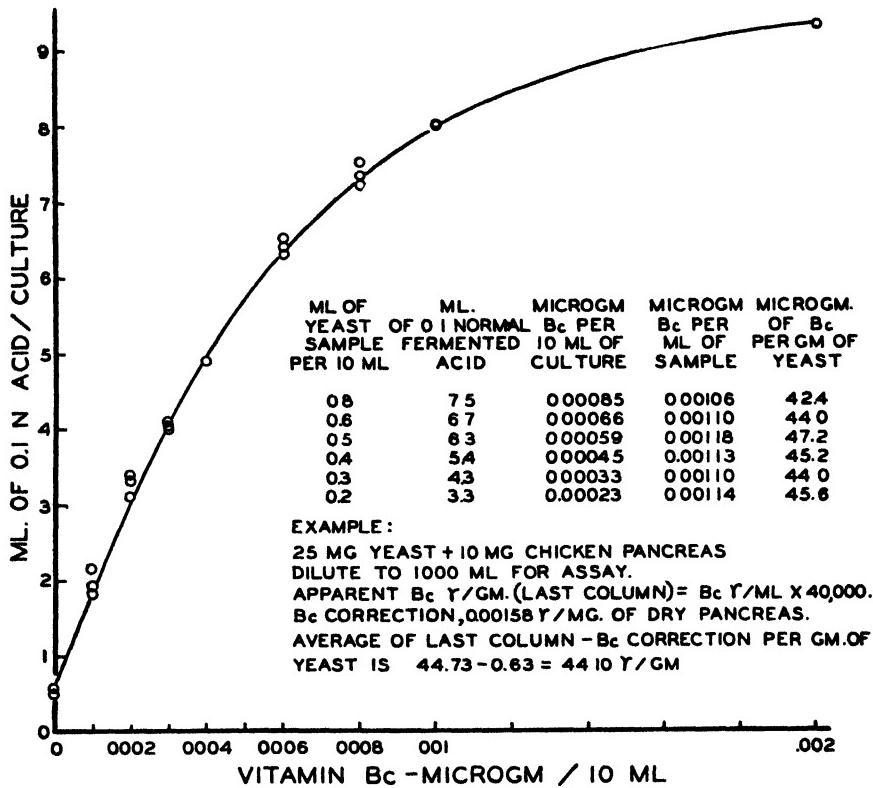


FIG. 1

Dosage-Response Curve Showing the Production of 0.1 N Acid by *L. casei* Cultures (Triplicated) in Relation to the Concentration of Pure Vitamin Bc per 10 ml. of Medium

The accompanying example of actual titration values obtained in an assay of pancreas-digested microorganisms illustrates the experimental design and method of calculating the final results.

to produce maximum acid in the standard series, has been found to result in the formation of the same maximum amount of acid as was obtained with excess pure Bc. This is a further indication that the basal medium is adequate for the assay of Bc contained in the products of these microorganisms. Comparable results were obtained in experiments with yeast and bacteria assayed with the *L. casei* medium proposed by Topley and Elvehjem (12) and with the medium used throughout the investigations reported in this paper. With a Bc dosage range from 0 to 0.002 γ per tube, we have found an almost linear response from 2.4 to 16.8 ml. of *N/10* acid in Topley and Elvehjem's medium. It seems probable that the wider dosage-response curve obtained with such a highly buffered nutrient solution containing peptone and a large amount of sugar would make the new medium proposed by Topley and Elvehjem very useful for assays with species of *Lactobacillus*.

#### *Liberation of Conjugated Vitamin Bc*

Preliminary studies on effective means of liberating Bc from type 3 yeast extract included acid and alkaline hydrolyses, and digestion with numerous kinds of enzyme preparations. The comparatively high values which were obtained consistently with samples of yeast treated with homogenized fresh chicken pancreas (see Table II) led to the routine use of this material in a survey of the vitamin Bc produced by microorganisms. Either fresh or dehydrated pancreas obtained from fowl may be employed as the source of enzymes. When used fresh, a pancreas weighing approximately 4 g. is ground in a glass mortar or in a Waring Blender, and suspended in *M/5* phosphate buffer at pH 5.0. Dried pancreas may be prepared by grinding the tissue in acetone, squeezing the fine material through cheesecloth, filtering, and rapidly drying the residue.\* For routine assays of total vitamin Bc in plant and animal tissues, it does not seem necessary to further purify the enzyme mixture. The defatted dried powder has been found to retain its activity for several months when stored at or near 0°C.

\* After this manuscript was written, the paper of Laskowski, Mims, and Day, *J. Biol. Chem.* **157**, 731-739 (1945), appeared with directions for partial purification of the enzyme of chicken pancreas.

TABLE II

*Vitamin Bc ("L. casei Factor") Determinations on Various Autolyzed Enzymes and on Type 3 Yeast Extract Digested under Benzene with These Enzymes at 37°C. in Phosphate Buffer Solution at pH 5.0 and 7.0*

Values are expressed as  $\gamma$  per g. of yeast extract (corrected for Bc in the enzyme) or per g. of autolyzed enzyme.

Enzyme material	pH 5.0	pH 7.0
Yeast + chicken pancreas (dehydrated)	52.5	—
Yeast + clarase	11.3	16.0
Yeast + hog kidney (dehydrated)	4.1	3.7
Yeast + mylase P	3.2	2.9
Yeast + multizyme	3.1	3.1
Yeast + pancreatin	2.9	3.0
Yeast + taka-diastase	2.7	3.1
Yeast + papain	3.2	3.4
Yeast + rhozyme S	3.5	4.0
Yeast + rhozyme Dx	15.3	13.6
Yeast + rapidase	3.2	3.3
Yeast + diastase of malt	5.5	3.2
Yeast + almond emulsin	9.7	3.0
Yeast extract, type 3 (autolyzed)	3.0	3.3
Chicken pancreas (autolyzed)	1.58	—
Clarase (autolyzed)	4.13	3.35
Hog kidney (autolyzed)	1.93	2.07
Mylase P (autolyzed)	0.24	0.22
Multizyme (autolyzed)	0.22	0.17
Pancreatin (autolyzed)	0.13	0.11
Taka-diastase (autolyzed)	0.88	0.75
Papain (autolyzed)	0.09	0.09
Rhozyme S (autolyzed)	3.73	0.10
Rhozyme Dx (autolyzed)	0.34	0.20
Rapidase (autolyzed)	<0.09	<0.09
Diastase of malt (autolyzed)	0.88	0.92
Almond emulsin (autolyzed)	3.40	1.79

Digestion may be performed with 25 mg. of dry yeast or an equivalent amount of wet sample contained in 20 ml. of phosphate buffer at pH 5.0, and 5 or 10 mg. of dry pancreas powder (fresh pancreas is approximately 44% dry matter) contained in 1 ml. of phosphate buffer. After thorough mixing, a small volume of benzene is added to minimize bacterial growth and the stoppered flasks are incubated at 37°C. for about 18 hours. Enzyme activity is stopped by steaming the samples at 100°C. for 10 minutes. The reaction is adjusted to pH 6.8, the solution made to some convenient volume, say 100 ml., and filtration accomplished with the aid of super-cel in Büchner funnels. Interfering fatty substances apparently are removed more satisfactorily by filtration than by extraction (15). A series of aliquots calculated to fall within the range of response is assayed in the usual manner.

The Bc content of the pancreas amounts to approximately  $0.0016 \gamma$  per mg. of dried pancreas, and hence the correction is usually small, amounting to about 1% of the vitamin contained in type 3 yeast extract when 10 mg. of dry pancreas are used to digest 25 mg. of the yeast powder. Advantages of pancreatic digestion are: (1) ready availability of live fowl, (2) ease of preparation of crude enzyme, (3) retention of activity when stored dry at low temperature, (4) effective liberation of Bc from conjugates, (5) close agreement of the bacterial assay with tests made on anemic chicks (16). A few determinations made on meat and vegetables indicate that the method may have general application in microbiological assay of vitamin Bc.

The results of an experiment designed to show the amounts of "*L. casei* factor" obtained in a free, active state when various complex natural mixtures are incubated together at pH 5.0 and 7.5 are presented in Fig. 2. The amounts of each kind of cellular extract employed in the phosphate buffer solution (21–23 ml. total volume) were as follows: powdered type 3 yeast extract, 25 mg.; fresh rat liver, 100 mg.; dry chicken pancreas, 10 mg.

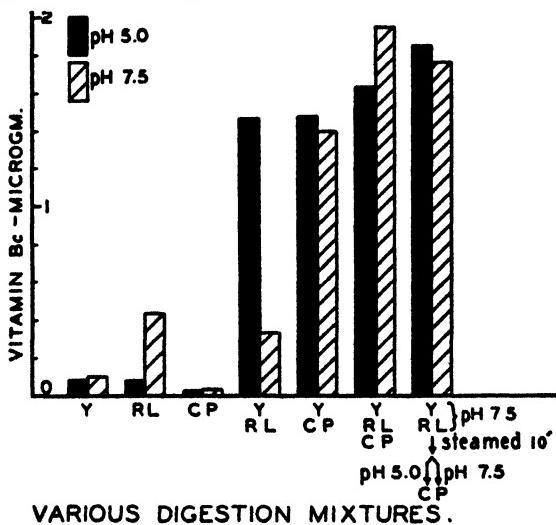


FIG. 2

## Assays of Vitamin Bc in Various Reaction Mixtures at pH 5.0 and 7.5

Y represents 25 mg. of type 3 yeast extract; RL, 100 mg. fresh rat liver; CP, 10 mg. dehydrated chicken pancreas. All suspensions were incubated under benzene at 37°C. for 20 hrs., except the experiment on the extreme right, where yeast and rat liver were incubated at pH 7.5 for  $7\frac{1}{2}$  hrs., then steamed, and divided into two equal parts, one being maintained at pH 7.5, the other adjusted to pH 5.0, and both digested with chicken pancreas for 20 hrs. See text for discussion.

The tissues were finely ground and suspended in water, and appropriate volumes were then pipetted into the phosphate buffer and incubated under benzene at 37°C. for 20 hours. The yeast, rat liver and chicken pancreas were autolyzed alone, and also allowed to digest together in various combinations. Also, yeast and rat liver were allowed to digest at pH 7.5 for 7½ hours, then steamed for 10 minutes, divided into two parts, and subsequently digested for 20 hrs. at pH 5.0 and 7.5 with chicken pancreas.

At either pH level, the autolyzed values for yeast and chicken pancreas were relatively low. In the case of rat liver, the autolyzed value was appreciably higher at pH 7.5 than at 5.0. In the mixture of yeast and rat liver, a large increase of the vitamin occurred at pH 5.0, but less was obtained at pH 7.5 than with liver alone. That liver enzymes do not destroy the vitamin at pH 7.5 is indicated by the results obtained with pancreatic digestion of the liver-yeast mixture which had been allowed to react for 7½ hrs. There is some evidence for the suggestion that the vitamin may be bound by liver extract at pH 7.5. The incubation of rat liver with a known amount of added vitamin Bc in phosphate buffer at pH 7.5 does not allow complete recovery of the vitamin, whereas incubation of a similar mixture at pH 5.0 permits recovery of approximately the entire amount of free Bc (see Table III).

An attempt has been made to show that the vitamin Bc in reaction mixtures of yeast, rat liver and chicken pancreas can be increased by addition of xanthopterin. These experiments were performed in the usual way by allowing ground homogenized cell materials to react under benzene at 37°C., and then assaying the filtrates with *L. casei*. The results are given in Table III. These data are not calculated on the basis of any particular substrate in the mixture because it seems much simpler to express the results in terms of total amounts of the active factor recovered from comparable mixtures. Although other investigators (7, 8) have reported substantial increases of "*S. lactis* R factor" with xanthopterin added to tissue extracts, we have been unable to find convincing evidence for the increase of "*L. casei* factor" in similar reaction mixtures. Furthermore, the addition of xanthopterin to synthetic medium used for the cultivation of four strains of bacteria and several yeasts yielded data which fail to indicate enhanced synthesis of Bc in the presence of this pterin.

The influence of pH on activity of pancreatic enzyme in the digestion process was studied in several experiments. Digestion of type 3 yeast

TABLE III

*Vitamin Bc ("L. casei Factor") Recovered from Different Reaction Mixtures  
in Phosphate Buffer With and Without Added Xanthopterin*

Values are given for the total reaction mixture

Fleischmann's type 3 yeast extract	Crystalline vitamin Bc	Fresh rat liver	Xanthopterin	Dried chicken pancreas	pH	Vol. of reaction mixture	"L. casei factor" recovered from mixture
	mg.	γ	mg.	γ	mg.	ml.	γ
			500		5.0	25.0	0.39
			500	100	5.0	25.0	0.59
			500		7.5	25.0	2.16
			500	100	7.5	25.0	1.90
25		100			5.0	22.0	1.46
25		100	20		5.0	22.1	1.48
25		100			7.5	22.0	0.34*
25		100	20		7.5	22.1	0.30*
	1	100			7.5	21.0	0.81
	1	100	20		7.5	21.1	0.76
	1	100			5.0	21.0	1.02
	1	100	20		5.0	21.1	0.98
	1			10	7.5	21.0	1.02
	1		20	10	7.5	21.1	1.12
	1			10	5.0	21.0	1.04
	1		20	10	5.0	21.1	0.95
				100	5.0	21.0	0.23
				100	7.5	21.0	0.25
25					5.0	21.0	0.08
25					7.5	21.0	0.09

\* These values are less than would have been obtained with rat liver alone.

extract with chicken pancreas was allowed to proceed over a wide range of pH values obtained with *M/5* phosphate buffer mixtures. Ten milligrams of fresh chicken pancreas were used with each 25 mg. of type 3 yeast extract powder in 25 ml. of buffer solution. The series of reaction mixtures were allowed to digest at 37°C. for 1/2, 1, and 3 hour periods. The reaction was stopped by boiling, and tests for Bc were then made in the usual way. Maximum release of the vitamin in these short-time experiments was observed repeatedly at about pH 4.5 to 5.0. The greatest activity for production of "folic acid" by rat liver in the presence of taka-diastase and xanthopterin has

been reported by other workers to occur at approximately pH 7.5 (8).\* It is possible that different chemical compounds are being acted upon at different rates over the pH range. Assays with both *S. lactis* and *L. casei* should be performed on the same samples in order to clarify the situation.

In experiments where reaction mixtures of yeast extract and chicken pancreas were kept at different temperature levels over the range from 0° to 100°C., marked decreases in yield of Bc were obtained above 62°C. Boiling the pancreas for several minutes destroyed its "Bc-conjugase" activity. Yields of Bc were found to be greatly diminished, also, where citrate was used instead of phosphate buffer. The inhibitory influence of citrate appears to operate in connection with the liberation of Bc from its conjugated form; incubation of pure Bc in citrate buffer at pH 5.0 had no destructive effect upon the vitamin, as indicated by assays with *L. casei*.

TABLE IV  
*Release of Vitamin Bc ("L. casei Factor") from Yeast Material by Enzymes of Chicken Tissues*

Values are expressed as γ of Bc per g. of fresh chicken tissue or dry yeast substrate.

Digestion mixture: Ground chicken tissue + yeast material  
in pH 5.0 phosphate buffer.†

Source of enzyme	Bc in fresh chicken tissue γ/g.	Bc in type 3 yeast extract γ/g.	Bc in type 200 B yeast cells γ/g.
Autolyzed samples	—	3.0	0.7
Gizzard lining	0.043	13.4	9.9
Upper intestinal mucosa	0.099	28.6	11.5
Lower intestinal mucosa	0.166	39.3	18.5
Pancreas	0.174	53.9	23.2

† Approximately 150 mg. of ground chicken tissues were used as sources of enzymes for digesting each 50 mg. sample of yeast in 20 ml. of buffer. Other experiments showed this amount of chicken pancreas to be greatly in excess. The yeast values have been corrected for Bc content of the enzyme preparation.

The comparative activity of enzyme mixtures obtained from different tissues of chicken was tested on Fleischmann type 200 B dried yeast cells and Fleischmann type 3 yeast extract powder. The analytical data, which were obtained with *L. casei*, are shown in

\* Laskowski, Mims, and Day, *J. Biol. Chem.* 157, 731-739 (1945), have reported maximum rate of release of "*S. lactis* R factor" at about pH 8.0 in reaction mixtures of yeast and chicken pancreas.

Table IV. Values for free Bc observed in the autolyzed samples are the lowest and the assays performed with pancreas yield the highest figures in the series. In the solutions of materials which were allowed to undergo pancreas digestion, the non-cellular yeast extract appears to contain about 2.7 times as much total Bc as the sample of type 200 B yeast cells, expressed on a dry weight basis. It is apparent that suitable digestion procedures are essential for the liberation of bound vitamin Bc in natural materials preliminary to the determination of the vitamin with microbiological assays.

### *Production of Vitamin Bc by Microorganisms*

Production of vitamin Bc by several hundred strains of bacteria, yeasts and molds has been determined with the chicken pancreas-*L. casei* method. The bacteria were grown in a medium (pH 7.0) composed of salts, dextrose, casein, amino acids, cystine, tryptophan, and vitamins, exclusive of Bc. The yeasts and molds were cultivated in a solution (pH 4.5) containing salts, glucose, asparagine, and vitamins, exclusive of Bc. The microorganisms were grown in pure culture on a shaking machine usually at 28°C. for periods varying from 3 to 10 days. Assays were performed on autolyzed and pancreas-digested whole cultures, including the organisms and the fermented liquor. A brief summary of the results is presented in Table V, together with data determined for some representative species in each group.

TABLE V  
*Production of Vitamin Bc ("L. casei Factor") by Microorganisms Grown  
in Chemically Defined Media*

Values are expressed as  $\gamma$  per ml. of homogenized culture unless stated otherwise. A blank means not determined. Digestion mixture: 5 ml. of microorganisms in their fermented medium, 5 ml. phosphate buffer, and 5 mg. of dry or 10 mg. of fresh chicken pancreas.

Organism	Autolyzed $\gamma/ml.$	Pancreas digested $\gamma/ml.$
<i>Bacteria</i>		
<i>Aerobacter aerogenes</i> (B-424)	—	0.07
<i>Alcaligenes faecalis</i>	0.09	0.15
<i>Chromobacterium violaceum</i>	0.11	0.25
<i>Bacillus vulgaris</i> 4529 strain	0.26	0.46
<i>Bacillus vulgaris</i> Ford strain	0.21	0.31
<i>Bacillus subtilis</i> 209 strain	0.24	0.33
<i>Bacillus subtilis</i> Jordan strain	0.03	0.07

TABLE V—(Continued)

Organism	Autolyzed γ/ml.	Pancreas digested γ/ml.
<i>Bacillus subtilis</i> Marburg strain	0.45	0.45
<i>Bacillus megatherium</i> N.Y.U. strain	0.09	0.10
<i>Proteus</i> X19	0.11	0.17
<i>Pseudomonas fluorescens</i>	0.10	0.14
<i>Serratia marcescens</i>	0.25	0.28
<i>Yeasts</i>		
<i>Fleischmann's</i> type 3 dry yeast extract	2.6 to 3.0 (γ/g.)	52.0 to 53.9 (γ/g.)
<i>Fleischmann's</i> 200 B dry yeast cells	0.7 (γ/g.)	23.2 (γ/g.)
<i>Candida guilliermondii</i> 309	—	0.05
<i>Candida guilliermondii</i> 311	—	0.33
<i>Candida flareri</i>	—	<0.01
<i>Kloeckera brevis</i>	—	0.36
<i>Saccharomyces cerevisiae</i> G.M.	—	0.12
<i>Saccharomyces cerevisiae</i> F.B.	—	0.18
<i>Saccharomyces ludwigi</i>	—	0.01
<i>Torulopsis dattila</i>	—	0.23
<i>Torulopsis pulcherrima</i>	—	0.14
<i>Torulopsis utilis</i>	—	0.06
<i>Fungi</i>		
<i>Aspergillus luteo-virescens</i>	—	<0.002
<i>Aspergillus niger</i>	—	0.035
<i>Aspergillus oryzae</i>	—	0.023
<i>Chaetomium ochraceum</i>	—	0.016
<i>Circinella sydowii</i>	—	0.012
<i>Fusarium sambucina</i>	—	0.013
<i>Mucor javanicus</i>	—	0.039
<i>Nectria peziza</i>	—	0.003
<i>Penicillium camemberti</i>	—	0.059
<i>Penicillium notatum</i>	—	0.096
<i>Penicillium roqueforti</i>	—	0.036
<i>Rhizopus microsporus</i>	—	0.090
<i>Rhizopus oryzae</i>	—	0.073
Summary of groups	No. of strains	Autolyzed range γ/ml. Pancreas digested range γ/ml.
Bacteria	42	<0.03 to 0.45
Yeasts	369	—
Fungi	84	—

Great variations were observed in the amounts of the vitamin produced by different species within each group of microorganisms. Fungi appear to be generally less productive than members of the other groups. In certain experiments with molds grown in 1% yeast

extract and 1% glucose, less Bc was actually recovered in pancreas-digested samples of the fermented liquor than was present in the uninoculated medium. Such indications of Bc destruction by molds were observed, for example, in cultures of *Absidia spinosa*, *Aspergillus niger*, *Circinella umbellata*, and *Rhizopus microsporus*. The liberation of Bc by naturally occurring enzymes in the microorganisms appeared to be greatest in certain bacteria; hence further studies were made on the Bc production by selected strains of bacteria cultivated in several kinds of media.

More intensive investigations centered around a few bacteria including several strains of the following: *Bacillus subtilis*, *B. vulgatus*, *Serratia marcescens*, and an unidentified Gram-negative rod isolated from the intestine of a fowl. It had been observed earlier in the course of this work that many bacteria and fungi grow well in a solution containing 1% yeast extract and 2% dextrose. This medium was employed, then, in a study of the ability of bacteria to release the conjugated vitamin contained in the medium, and in addition produce some additional Bc in their own anabolism. Other nutrient media, also, were formulated to contain suitable substrate materials conducive to growth of the bacteria and production of free vitamin Bc on a large scale. Such media included inorganic salts, autoclaved cells of bakers' yeast, bran, ground whole wheat or ground whole barley, together with varying amounts of dextrose from 1 to 6%. The bacteria were grown in flasks on a shaker at 25°C. for periods up to 7 days. After further autolysis under benzene for 18 hours, the cultures were filtered through super-cel and the liquid was then assayed in the customary manner. Not all bacteria are filtered out by super-cel, and it may be that high speed centrifugation would provide a better sample of the solution. Filtration through a Seitz disc was found unsatisfactory because Bc was removed from the sample.

Some of the results shown in accompanying Table VI are typical of the data obtained in experiments with bacteria grown in liquid media containing varied amounts of yeast extract and dextrose. The yields vary in the different media up to about 1.67 γ of Bc per ml. of filtered autolyzate, and up to approximately 100 γ per g. of yeast material used in the medium. In the 2% yeast extract medium, containing initially about 0.06 γ of free Bc and 1.04 γ of total Bc per ml. the activity of the different bacteria resulted in yields of 0.48

TABLE VI

*Production of Free Vitamin Bc ("L. casei Factor") in Media Containing Varied Amounts of Yeast Material and Dextrose*

Bc values are expressed as  $\gamma$  per ml. of autolyzed and filtered culture solution.

Organism	Type 3 yeast extract medium				Type 200 B yeast cells medium  2% yeast 2% dextrose
	1% yeast 2% dextrose	1% yeast 4% dextrose	2% yeast 4% dextrose	5% yeast 4% dextrose	
<i>Bacillus subtilis</i> (Marburg strain)	0.67	0.72	0.48	0.94	0.60
<i>Bacillus vulgaris</i> (Ford strain)	0.25	0.30	0.63	0.50	0.39
<i>Serratia marcescens</i>	0.61	0.58	1.33	1.52	0.31
<i>Chicken bacterium</i>	0.98	0.98	1.67	1.60	0.71

to 1.67  $\gamma$  of free Bc per ml. in the autolyzed cultures. In the 2% yeast cell medium (Fleischmann's type 200 B dried yeast) containing 0.013  $\gamma$  of free Bc and 0.59  $\gamma$  of total Bc per ml., the bacterial autolyzates yielded from 0.31 to 0.71  $\gamma$  per ml. In other experiments with *B. subtilis* (Marburg) and the chicken bacterium, grown at 28°C. in yeast extract medium for 3, 5 and 7 days, it was found that the largest yields occurred in cultures grown for about 3 days. Some representative values for Bc, expressed as  $\gamma$  per ml. of fermented culture, are as follows: autolyzed cultures, 3 days 0.60, 5 days 0.37, 7 days 0.44; pancreas-digested cultures, 3 days 0.72, 5 days 0.66, 7 days 0.62. The values obtained with the chicken bacterium are as follows: autolyzed cultures, 3 days 0.81, 5 days 0.75, 7 days 0.60; pancreas-digested cultures, 3 days 0.78, 5 days 0.73, 7 days 0.56. The significance of these data lies in the discovery that some bacteria not only produce certain amounts of vitamin Bc but that they are able to release this vitamin in an active form which stimulates growth of the assay bacteria.

Further studies were undertaken with bacteria grown for 4 days at 28°C. in media containing different concentrations of Fleischmann type 3 yeast extract with or without added dextrose. The whole cultures were then digested under benzene at 37°C. and pH 5.0 for 18 hours. Assays were made with *L. casei*, using Topley and Elvehjem's medium (12). The media used for Bc production were made by dis-

solving from 0.5 to 5.0 g. of dry yeast extract in distilled water and adjusting the pH to 7.0. The Bc values obtained in the different culture solutions are shown in Table VII. It is apparent that the yield of free Bc is proportional to the amount of yeast extract present in the medium. The addition of dextrose seems to increase the yield of Bc somewhat at the lower levels of yeast supplied. In a medium containing 3% yeast extract, 52 γ of free Bc were found per g. of yeast extract without added sugar, and 79 γ per g. of yeast extract when 2% dextrose was supplied in the medium. In 5% yeast extract medium, the yield was 48 γ of Bc with or without added dextrose.

TABLE VII

*Production of Free Vitamin Bc ("L. casei Factor") by Serratia marcescens in Media Containing Varied Amounts of Yeast Type 3 Extract and Dextrose*

Bc values, determined with Topley and Elvehjem's medium (12), are expressed as γ per ml. of autolyzed and filtered culture solution.

Yeast extract Dextrose	0.5%	1.0%	3.0%	5.0%
0	0.19	0.42	1.57	2.41
0.5%	0.41	0.51	1.64	2.21
1.0%	0.42	0.65	1.96	2.49
2.0%	0.20	0.78	2.39	2.38
4.0%	0.18	1.18	1.48	1.95
6.0%	0.21	0.40	1.48	2.35

In another experiment, 40 cultures of unidentified soil bacteria were grown in a medium containing 2% yeast extract and 2% dextrose. The yields of Bc obtained in the autolyzate varied from 0.05 to 1.9 γ per ml. Some of these organisms grow rapidly and appear to be very active in releasing Bc from yeast extract. *Serratia marcescens* was found to yield 1.25 γ of Bc per ml. of autolyzed medium, when grown in 4% wheat bran and 2% dextrose solution. Further experiments with other bacteria cultivated in various media are in progress.

Since the vitamin Bc data obtained with pancreas-digested samples of yeast materials tested in *L. casei* titrimetric assays show close agreement with values obtained with anemic chicks, it appears likely that such microbiological determinations on various natural materials may represent the total amounts of the vitamin available for animal nutrition. The possible role of pancreatic enzymes *in vivo* for the liberation of conjugated vitamin Bc in the intestinal tract of animals seems to have some significance in nutrition studies.

Although enzymes of animal origin show high activity in liberating conjugated Bc from crude materials, the enzymes of certain kinds of bacteria would seem to offer distinct advantages for the production of free vitamin Bc in large scale commercial operations. The use of yeasts or cereal grains as substrates for the metabolic activities of certain bacteria, which are rich in vitamin "Bc conjugase," should provide large amounts of free vitamin Bc and other vitamins in a solution which can be concentrated and dehydrated in order to produce preparations of high vitamin potency.

#### SUMMARY

Determinations of total vitamin Bc activity by means of the *Lactobacillus casei* assay method were made on autolyzed and enzyme-digested cultures of 82 strains of bacteria, 369 yeasts and 84 molds. A crude enzyme mixture prepared from chicken pancreas was found most satisfactory among all methods tried for the release of conjugated Bc in microorganisms. No evidence was found for increase of Bc activity in reaction mixtures of rat liver or chicken pancreas to which xanthopterin was added, and supplying this pterin to cultures of bacteria and yeasts failed to stimulate production of the vitamin. Certain strains of bacteria which were found to produce considerable amounts of vitamin Bc also showed considerable Bc conjugase activity. It is suggested that selected microorganisms may be useful for large scale production of Bc concentrates.

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# Absorption Spectrum of $\zeta$ -Carotene<sup>1</sup>

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Received March 31, 1945

## INTRODUCTION

During the last few years attention has been called several times to a carotene or carotenes having absorption maxima in petroleum ether near 4000 and 4250 Å. van Stolk, Guilbert, and Penau (1) predicted in 1931 the occurrence of such a pigment on the basis of high light absorption at 4046 and 4280 Å shown by the mother liquors from  $\beta$ -carotene crystallization. In 1939 Strain (2) prepared a pigment from carrots having maxima at 4000 and 4250 Å. White, Zscheile and Brunson (3) confirmed Strain's work on this pigment and reported maxima at 3600, 3790, 4000 and 4250 Å. They also studied a pigment from corn, the spectrum of which had an additional minor maximum at 4520 Å, which may have been due to a contaminant. A fraction having a similar spectrum was prepared from yellow corn by Baumgarten, Bauernfeind and Boruff (4). The K-carotene described by Fraps and Kemmerer (5) differs from the carotene described in this paper in its absorption maximum at 4500 Å and in its failure to show a maximum at 3800 Å. Strain and Manning (6) in 1943 provisionally applied the name  $\zeta$ -carotene to a carrot pigment with maxima at 4000 and 4250 Å.

The carotene described in this paper was prepared from carrots as well as from tomatoes and the two preparations were shown to have identical characteristic absorption curves. The similarities of occurrence, absorption spectrum and relative position on a magnesia

<sup>1</sup> Journal Paper No. 209 Purdue University Agricultural Experiment Station. Studies on Carotenoids No. 8.

<sup>2</sup> Ensign U.S.N.R. This article is not intended to reflect official views of the Navy Department.

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column indicate that the pigment is identical with the  $\zeta$ -carotene described by Strain. The name  $\zeta$ -carotene is, therefore used in this paper.

The authors have obtained spectroscopic evidence of the presence of  $\zeta$ -carotene in butter, yellow beef fat and eggs, as well as in certain strains of tomatoes and carrots.

## METHODS

### *Preparation of Solutions of $\zeta$ -Carotene*

Fruits of two special tomato selections developed at the Purdue Agricultural Experiment Station were used as source material for the preparation of  $\zeta$ -carotene. These tomatoes have especially high  $\zeta$ -carotene contents.

A bushel of tomatoes was ground to a pulp in a meat grinder and partially dehydrated by adding three gallons of 95% ethanol. After an hour the alcohol had caused sufficient flocculation of the solid matter to permit straining through a cheese cloth. The solid residue was extracted by stirring two hours with three gallons of acetone and two gallons of petroleum ether (b.p. 40-60°). Two such extractions were necessary to remove substantially all the color. The petroleum ether fraction was washed several times with water, then concentrated to four liters under reduced pressure and again washed six or seven times with water to remove the last traces of acetone. A preliminary separation of the  $\zeta$ -carotene from the other pigments present was obtained by chromatography of the extract on a 50% magnesia-supercl mixture. The  $\zeta$ -carotene occurred on the chromatogram below  $\gamma$ -carotene and just above  $\beta$ -carotene. Hexane was used in developing the column, and hexane containing 10% ethanol in eluting the  $\zeta$ -carotene zone. (The hexane used in this and subsequent steps was purified by running it through a silica gel column (7) after distillation.) A second chromatogram on magnesia-supercl followed by a third on alumina gave a preparation which could not be further purified by use of these adsorbents. The alumina columns were developed with hexane containing 2.5% ethyl ether.

### *Determination of Specific Absorption Coefficients*

Attempts to crystallize the pigment were unsuccessful. Repeated efforts to effect crystallization from benzene by addition of methanol, from toluene by addition of methanol and cooling to dry ice temperatures, and from hexane by addition of ethanol and cooling led only to the formation of an impure curdy product. Rapid oxidation of  $\zeta$ -carotene in polar solvents, a fact noted by Strain (2), and the presence of a colorless impurity apparently were responsible for the heterogeneity of the product obtained.

This colorless impurity occurred in much larger amounts in the  $\beta$ -carotene fraction from the magnesia columns and an investigation of its nature was therefore undertaken in the  $\beta$ -carotene fraction. The impurity was not removed by chromatography on magnesia, calcium hydroxide, or alumina, nor by mild saponification. Boiling 8 hours in a 2.5 N solution of sodium ethylate in ethanol followed by extraction with 95% methanol and chromatographic adsorption on alumina did remove nearly all of the impurity from the  $\beta$ -carotene fraction. Since  $\zeta$ -carotene preparations with specific absorption coefficients (alpha values<sup>4</sup>) of 120 and 186 before sodium ethylate saponification had alpha values of 198 and 204 respectively, after this treatment, it was assumed that the impurities were removed. Longer saponification periods did not effect any further apparent purification. The behavior of the impurity on a chromatogram and the difficulty of saponification indicated that the substance probably was a wax. A Liebermann-Burchard test of this substance was negative.

Crystallization of  $\zeta$ -carotene in the absence of the wax has not been extensively investigated, but temporary interruption of this work by the military service of one of the authors has led them to publish the values obtained by weighing non-crystalline residues. These values may be modified when and if crystalline material becomes available.

In a typical experiment to determine specific absorption coefficients, 40 ml. of a solution containing approximately 30 mg. of  $\zeta$ -carotene was refluxed four hours with 100 ml. of 2.5 N sodium ethylate solution in ethanol. The solution was transferred to a separatory funnel and 15 ml. of water and 50 ml. of hexane added to effect phasic separation. The aqueous phase was discarded, 20 ml. of 20% KOH in methanol was added to the hexane solution and the mixture refluxed 30 minutes. After draining off the KOH solution, the hexane solution was extracted four times with 100 ml. portions of 95% methanol and then washed free of methanol by repeated washing with water. The entire saponification and extraction procedure was repeated on the same sample.

The solution from the second saponification was chromatographed on alumina. Hexane containing 3.5% ethyl ether was used as a developing agent. The slightly colored portion above the main zone of  $\zeta$ -carotene and the lower one-fourth of the  $\zeta$ -carotene band were discarded to limit the inclusion of isomers caused by the

$$\log_{10} \frac{I_0}{I}$$

<sup>4</sup> Specific absorption coefficient,  $\alpha = \frac{\log_{10} \frac{I_0}{I}}{cl}$ ;  $I_0$  = intensity of radiant energy transmitted by solvent-filled cell,  $I$  = intensity transmitted by solution-filled cell,  $c$  = concentration in grams/liter and  $l$  = thickness of layer in cm.

heating during saponification. Elution was accomplished with hexane and ethyl ether. The resulting solution was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and filtered through sintered glass.

The weight of  $\zeta$ -carotene in the resulting solution was determined as follows: the solution was evaporated to dryness in a stream of  $\text{CO}_2$  at reduced pressure and further dried in vacuo overnight at 12 microns pressure; the container and carotene were weighed, the carotene was removed with hexane and the container reweighed after drying. These operations were all accomplished with a minimum of exposure to oxygen, in a special container designed to keep the sample in a  $\text{CO}_2$  atmosphere. The hexane solution of the pigment was made to volume and its absorption curve determined by a method previously described (8).

### *Correction of Absorption Curve for Isomerization*

The heating incidental to saponification caused some isomerization of the carotene as shown by a raising of the absorption curve at the wave lengths below 3500 Å and a dropping of the curve at longer wave lengths. The presence of at least one isomer was confirmed by repeated chromatography of the lower portion of the  $\zeta$ -carotene zone from an alumina chromatogram of such a mixture. A fraction was obtained which had maxima shifted 20 Å toward the ultraviolet. Addition of iodine to a solution of this fraction and exposure to light caused the maxima to be shifted back to longer wave lengths, 3995 and 4240 Å.

It was found that refluxing of a hexane solution of  $\zeta$ -carotene caused no change in the absorption at 3560 Å. This point was therefore selected as a coincident point for the all-*trans* isomer and the isomerization mixture. Assuming that the preparation chromatographed without saponification represented the all-*trans* isomer and assuming the isomerization mixture and the all-*trans* isomer to have identical absorption values at 3560 Å, specific absorption values for the all-*trans* isomer were calculated at other wave lengths from the characteristic curve.

### RESULTS

Curve I of Fig. 1 represents specific absorption coefficients of  $\zeta$ -carotene in hexane solution as determined experimentally. The specific absorption values of the all-*trans* form, as corrected by the coincident point technique previously described, are represented by Curve II in Fig. 1. The values plotted represent a single determination. Replicate determinations gave satisfactory checks, as shown by Table I.

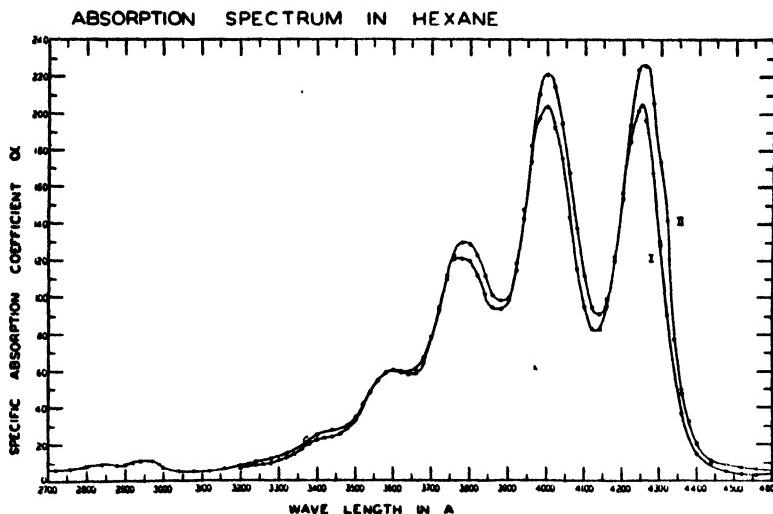


FIG. 1

Absorption Spectrum of  $\beta$ -Carotene in Hexane  
 Curve I represents experimentally determined values and Curve II represents values obtained for the all-trans isomer by correcting the experimental values for isomerization.

In Table II are tabulated the specific absorption values at maxima and minima as determined experimentally and after correction for isomerization.

Figure 2 shows the far ultraviolet absorption spectrum in iso-octane (2,2,4-trimethylpentane) of the preparation with the lowest cis peak and therefore the least isomerization. On refluxing this solution in iso-octane ten hours (99°), the specific values at the 2950 Å maximum increased to 21.7 and at the 2840 Å maximum to 18.8. The values

TABLE I  
*Replicate Determinations of Absorption Coefficients*

Determination	Experimental $\alpha$ -values (Curve I)		$\alpha$ -Values corrected for isomerization (Curve II)	
	3560 Å	4000 Å	3560 Å	4000 Å
1	54.8	204	54.3	221
2	54.1	198	54.3	219
3	54.0	204	54.3	219

TABLE II  
*Specific Absorption Coefficients of  $\zeta$ -Carotene at Maxima and Minima*  
(Minima italicized)

	Wave length in Angstrom units	Experimental $\alpha$ -values	$\alpha$ -Values corrected for isomerization
In hexane solution	4250	205	226
	4130	82.0	—
	4140	—	91.5
	4000	204	221
	3880	97.0	98.6
	3780	121	130
	3640	59.7	58.6
	3600	60.5	60.3
	3080	5.2	—
	2950	11.6	—
In iso-octane solution	2885	8.7	—
	2850	9.7	—
	3080	—	4.65
	2950	—	9.90
	2880	—	7.86
	2840	—	8.86
	2600	—	4.96
	2360	—	25.6

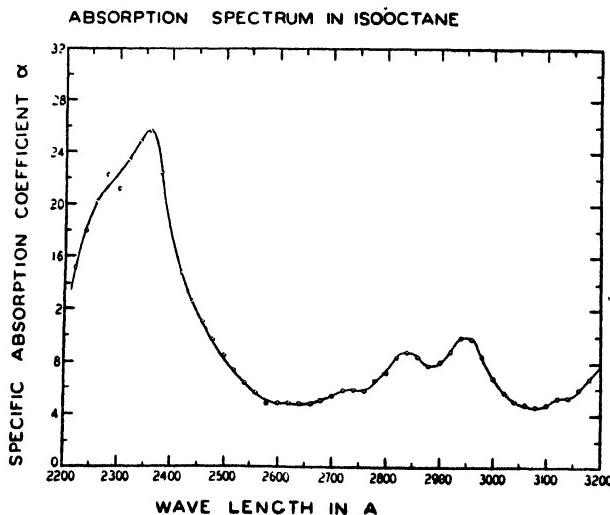


FIG. 2  
Absorption Spectrum of  $\zeta$ -Carotene in Iso-octane (2,2,4-Trimethylpentane)

at 3020 and 2600 Å and at the 2360 Å maximum showed little change. Thus the so-called *cis* peak increased on isomerization of the all-*trans* form as was pointed out earlier (9). The *cis* peak maxima shown by the absorption curve presented in Fig. 2 for the preparation showing the least isomerization are distinct enough to suggest that even in this preparation some isomerization had taken place.

The similarity in the shapes of the absorption curves of all-*trans*  $\zeta$ -carotene and lycopene, both in the visible and at the *cis* peak, is striking enough to suggest structural similarities. Similarities between the absorption curves in the *cis* peak region after isomerization are particularly striking.

### SUMMARY

$\zeta$ -Carotene has been prepared in solution by extraction of fruits from special tomato strains. After repeated chromatography and a saponification procedure to remove a waxy impurity, specific absorption coefficients were calculated from weight data obtained by evaporating the solutions to dryness in a CO<sub>2</sub> atmosphere. Absorption curves and specific absorption coefficients are presented. This carotene exhibits typical isomerization phenomena and the shape of its absorption curve is similar to that of lycopene. It may occur in nature in a wide variety of sources.

### ACKNOWLEDGMENTS

The authors wish to express appreciation to Dr. F. W. Quackenbush and Dr. J. W. Porter for generous advice and assistance in carrying out this work and to Dr. R. M. Caldwell and other members of the Department of Botany and Plant Pathology for the tomatoes.

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# The Amino Acids Yielded by Various Yeasts After Hydrolysis of the Fat-Free Material A Comparative Investigation

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Received March 19, 1945

## INTRODUCTION

Since the studies of Osborne and Mendel (1), it has been generally recognized that yeasts are not only valuable sources of the B vitamins but also furnish protein of high biological value. However, there are certain incompatibilities in the published results concerning the value of yeast proteins. The biological value of a protein is a composite of two major factors: one, amino acid composition of the intact protein; and two, digestibility of the protein and assimilability of the resulting split products. The interdependence of these two factors has been recently discussed by Mitchell (2). In this paper, we are concerned only with essential amino acid composition.

## EXPERIMENTAL

Ten samples of yeast and derivatives were obtained through the courtesy of Anheuser-Busch, Inc. These were selected to represent the widest possible range of environmental and genetic history, but were limited to types which are commercially important and available. A brief description of the yeast types and the methods used in producing them follows.

Strain K (105). This is a pure debittered brewers' yeast. It is a by-product of beer making and is grown on a cereal wort composed of extractives from 70% barley malt and 30% brewers' rice. 150 lbs. of dry substance of this cereal mixture ultimately accounts for the production of 1 pound of debittered yeast. Debittering does not change the nature of the yeast. The yeast is dried at approximately 95°C.

Strain G<sub>1</sub> (106). This is a debittered brewers' yeast regrown under primary yeast culture conditions in a hop-free medium composed of either beet molasses or cane molasses and corn extract. It was harvested after a single generation or after the weight had doubled. Nitrogen is supplied from two sources, approximately one third is organic N from corn extract, the remainder is inorganic N, either  $(\text{NH}_4)_2\text{SO}_4$  or  $\text{NH}_4\text{OH}$ .

Strain G<sub>3</sub> (107). This is the same yeast as strain G<sub>1</sub>, but regrown under primary yeast culture conditions for three generations in the molasses-corn extract-ammonia medium described under G<sub>1</sub>.

Strain B<sub>2</sub> (108). This is a primary grown strain of *Saccharomyces cerevisiae* brewers' type regrown for two cycles from freshly cultured seed yeast in a hop-free medium consisting of molasses, corn extract and ammonium salts. During two cycles approximately 100 pounds of seed yeast are expanded to about 8000 pounds of Strain B<sub>2</sub>. This yeast seed is started from pure culture in the laboratory and is grown for yeast yield alone rather than beer production or other fermentation by-products.

Strain B<sub>3</sub> (109). This represents the third cycle of growth for the primary *Saccharomyces cerevisiae* described under Strain B<sub>2</sub>. The medium is the same as previously described. The 8000 pounds of B<sub>2</sub> seed yield approximately 30,000 pounds of B<sub>3</sub>.

Kitchen Food Yeast (110). This food yeast is prepared from Strain B<sub>3</sub> by a partial autolysis using about 5% NaCl to aid the autolysis and to improve the flavor.

Autolyzed Yeast (111). This product is a mixture of 90% Strain G<sub>3</sub> and 10% Strain K which is allowed to autolyze in the presence of NaCl. After autolysis is complete the liquefied portion is separated and dried. The dry material is composed of approximately 50% yeast solubles and 50% NaCl.

Liquid Yeast Extract No. 3 (112). This is a liquid yeast extract prepared from Strain G<sub>2</sub> by autolysis in the presence of an organic solvent. The solubilized fraction is taken up in alcohol and the solvent then removed by concentration *in vacuo*. Approximately 1 lb. of dry extract is produced from 12 lbs. of dried yeast.

Strain A.M. (113). This yeast is a bakers' type strain of *Saccharomyces cerevisiae* grown in a medium in which all the nitrogen requirements were met by ammonium salts.

Strain A.M.<sub>2</sub> (114). This is the same strain as A.M. but grown in a medium in which one-third of the nitrogen requirements were met from organic corn extract sources.

The yeasts, except strain L.Y.E., were thoroughly extracted with warm acetone, warm benzene, and ether to remove lipid material. The air-dried yeasts were then dried in the oven at 80°C. for 24 hours. The following analytical methods were employed: nitrogen by micro Kjeldahl; sulfur by Pregl<sup>1</sup>; arginine, histidine, and lysine by a small scale modification of the Kossel-Kutscher method (3) using 8 N  $\text{H}_2\text{SO}_4$  for hydrolysis; tyrosine and tryptophan by Lugg's adaptation of the Millon reaction following hydrolysis with 5 N NaOH (3); and phenylalanine by a modification of the Kapeller-Adler procedure (3) using either 5 N NaOH or 7 N  $\text{H}_2\text{SO}_4$  for hydrolysis. Cystine was estimated by both the Folin and Fleming-Vassal methods (cf. 3) after hydrolysis with a 1:1 mixture of 18% HCl and 88% HCOOH. Methionine was estimated by the McCarthy-Sullivan method (cf. 3) after hydrolysis with

<sup>1</sup> Sulfur analyses are difficult to carry out on dried yeasts and may at times be low.

18% HCl, while threonine was calculated from the yield of acetaldehyde obtained after periodate oxidation (3). In this case, the HCl hydrolyzates were evaporated to dryness several times using freshly distilled water to eliminate any trace of alcohol or other solvent which might give  $\text{CH}_3\text{CHO}$  on treatment with periodate. Leucine, isoleucine, and valine were determined by an adaptation of the microbiological procedures of Kuiken, Norman, Lyman, Hale, and Blotter (4), of Shankman (5), and of McMahan and Snell (6).

To stress the *comparative* aspects of this study each amino acid, in all ten samples, was assayed simultaneously or consecutively by the same analyst with every effort being made to keep all conditions as constant as possible.

As we have repeatedly pointed out (cf. 3 and earlier papers), amino acid determinations carried out on hydrolysates of impure proteins prepared by boiling with strong acids or alkalies leave much to be desired. The methods, too, are probably better adapted to yield comparative results rather than absolute values. Thus, the isolation of arginine, histidine and lysine, especially the latter, can be expected to give minimal results only. The Millon-Lugg methods for tyrosine and tryptophan may be expected to be quite accurate. The errors in the Kapeller-Adler estimations for phenylalanine have been discussed in (7) and (3). As cystine is partly destroyed during hydrolysis, particularly in the presence of carbohydrate impurities, recovery experiments were carried out with each yeast. The accuracy of the colorimetric method for methionine and the oxidative procedure for threonine has been discussed previously (3). Although the microbiological methods may yield quite accurate results with some purified proteins, when applied to impure biological material such as yeast hydrolysates, they suffer from the same disadvantages as the chemical methods plus the added difficulty that certain B vitamins and other cellular constituents may have an influence on bacterial growth. Thus, Stokes and Gunness (8) have reported that the presence or absence of pyridoxamine determines the essentiality of lysine, threonine, and alanine for the growth of certain lactobacilli. Whether the microbiological estimations of leucine, isoleucine, and valine are subject to similar possibilities of error must await further study.

## RESULTS

The analytical results are summarized in Tables I and II. The amino acid values for strains A.Y. and L.Y.E. may be omitted from

TABLE I  
*Approximate Percentage Amino Acid Distribution in Fat Extracted Yeasts*

Strain	K 105	G <sub>1</sub> 106	G <sub>2</sub> 107	B <sub>2</sub> 108	B <sub>1</sub> 109	KFY 110	AY 111	LYE 112	AM 113	AM <sub>2</sub> 114
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	mg./cc.	per cent	per cent
Nitrogen	8.28	8.32	7.81	7.38	7.02	6.61	5.93	74.12	7.90	7.60
Sulfur	0.44	0.41	0.35	0.36	0.35		0.64		0.34	0.35
Arginine	2.72	2.70	2.08	1.41	2.05	1.66	1.78	14.02	1.67	1.96
Histidine	1.34	1.22	1.58	1.47	1.15	1.07	0.89	8.59	1.40	1.40
Lysine	3.48	3.55	3.69	3.74	3.55	3.31	1.83	21.00	3.75	3.29
Tyrosine	1.81	1.83	1.78	1.82	1.60	1.42	1.19	9.31	1.78	1.77
Tryptophan	0.78	0.75	0.60	0.61	0.56	0.48	0.43	3.87	0.66	0.63
Phenylalanine	2.40	2.27	1.97	1.91	2.04	1.09	1.13	17.55	2.21	1.86
Cystine	0.46	0.50	0.50	0.41	0.40	0.44	0.46	3.88	0.46	0.46
Methionine	1.47	1.38	1.32	1.26	1.15	1.15	0.78	9.49	1.36	1.34
Threonine	2.76	2.73	2.93	2.77	2.33	2.10	1.91	9.09	2.75	2.70
Leucine	3.67	4.41	3.56	3.74	3.25	2.81	2.52	20.47	3.01	3.80
Isoleucine	3.11	3.14	2.69	2.86	2.46	2.40	2.04	18.15	2.86	2.95
Valine	2.43	2.51	2.25	2.49	2.59	2.23	1.89	21.87	2.32	2.19
Cystine S/TS	28	33	37	31	30			19		34
Methionine S/TS	71	62	69	64	70			26		84

TABLE II  
*Approximate Percentage Amino Acid Distribution in Yeasts*  
(Calculated to 16.0 per cent of Nitrogen.)

Strain	K 105	G <sub>1</sub> 106	G <sub>2</sub> 107	B <sub>2</sub> 108	B <sub>1</sub> 109	KFY 110	AY* 111	LYE* 112	AM 113	AM <sub>2</sub> 114	Av. Values
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Arginine	5.3	5.2	4.3	3.1	4.7	4.0	4.8	3.0	3.5	4.1	4.3
Histidine	3.0	2.7	2.8	3.1	3.0	2.3	2.8	2.3	2.7	3.0	2.8
Lysine	6.7	6.8	7.6	8.1	8.1	8.0	4.9	4.0	7.6	6.9	7.5
Tyrosine	3.5	3.5	3.6	3.9	3.6	3.4	3.2	2.0	3.6	3.7	3.6
Tryptophan	1.5	1.4	1.2	1.3	1.3	1.2	1.1	0.8	1.3	1.3	1.3
Phenylalanine	4.6	4.4	4.0	4.1	4.2	2.9	3.0	3.9	4.5	3.9	4.1
Cystine	0.9	1.0	1.0	0.9	0.9	1.1	1.2	0.8	0.9	1.0	1.0
Methionine	2.8	2.7	2.7	2.7	2.6	2.8	2.1	2.1	2.7	2.8	2.7
Threonine	5.3	5.2	6.0	6.0	5.3	5.1	5.1	2.0	5.6	5.7	5.5
Leucine	7.1	8.5	7.3	8.1	7.4	6.8	6.8	4.4	6.1	8.0	7.4
Isoleucine	6.0	6.0	5.5	6.2	5.6	5.8	5.5	3.9	5.8	6.2	5.9
Valine	4.7	4.8	4.6	5.4	5.9	5.4	5.1	4.7	4.7	4.6	5.0

\* Omitted from the average.

comparison with the other yeasts as these autolyzed preparations represent only a portion of the original material.

Arginine. Strains K and G<sub>1</sub> appear to yield somewhat more arginine than the other yeasts.

Histidine. There is probably little difference between the histidine contents of the various strains except that the lower amount found in K.F.Y. appears to be significant. K.F.Y. is a partially autolyzed yeast. This processing may have led to some destruction of histidine or in some modification of the protein which resulted in a partial destruction of histidine during acid hydrolysis (cf. 9).

Lysine. Yeast proteins are an excellent source of this amino acid, strains B<sub>2</sub>, B<sub>3</sub> and K.F.Y. being especially valuable.

Tyrosine. There are no marked variations although the 0.5% difference between K.F.Y. and B<sub>2</sub> may be significant because of the great accuracy of the Lugg method.

Tryptophan. The distribution of this amino acid is similar to tyrosine. Strains K and G<sub>1</sub> appear to yield a little more of this essential amino acid than the others.

Phenylalanine. K.F.Y. yields distinctly less phenylalanine than the seven other strains.

Cystine and Methionine. These two sulfur containing amino acids are present in about the same ratios for all the strains and seem to account for approximately all the sulfur in the non-autolyzed yeasts.

Threonine. There does not appear to be any significant difference in the threonine yielded by the various strains.

Leucine, Isoleucine, and Valine. Although the values given in the tables represent the average result of ten culture tubes for each determination, the microbiological method is still too new to allow an opinion as to whether or not the apparent differences are significant.

## DISCUSSION

The inherent error in calculating protein from nitrogen, especially in certain plants such as yeasts, has long been known. If the plant proteins are isolated in the purified form and then analyzed for their constituent amino acids, and if the results so obtained are used to calculate the amino acid composition of the plant from its nitrogen content, the error is often quite considerable. But if the tissue is itself analyzed for the amino acids this error is minimized. Results so

obtained show the approximate amino acid composition of the proteinaceous substance irrespective of the presence of non-protein nitrogen which, in the case of yeast, may amount to 20% or more of the total nitrogen. The values in the tables, therefore, indicate the quantities of each of the essential amino acids which would be ingested by the consumption of 100 g. of yeast (Table I) or 16 g. of yeast nitrogen (Table II). The nature and quantity of the non-protein nitrogen, therefore, becomes irrelevant.

TABLE III  
*Approximate Amino Acids in Some Plant and Animal Proteins*  
(All Calculated to 16.0 per cent of Nitrogen.)

Amino Acid	Yeasts		Ave.	Yeast (10)	Yeasts (11, 12)	Meat (3, 13)	Casein (3)	White Flour (3, 4)	Corn Gluten (3)	Pol- ished Rice (14)
	Max.	Min.								
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Arginine	5.3	3.1	4.3	3.1	5.4	7.7	4.1	3.9	3.1	7.2
Histidine	3.1	2.3	2.8	3.3	3.1	2.9	2.5	2.2	1.6	1.5
Lysine	8.1	6.7	7.5	7.1 <sup>a</sup>	9.8	7.2	7.5	1.9	0.8	3.2
Tyrosine	3.7	3.4	3.6	3.8	6.0	3.4	6.4	3.8	6.7	5.6
Tryptophan	1.5	1.2	1.3	1.2	1.5	1.3	1.2	1.3	0.7	1.3
Phenylalanine	4.6	2.9	4.1	4.5		4.9	5.2	5.5	6.4	6.7
Cystine	1.1	0.9	1.0	1.1	2.3	1.3	0.4	1.9	1.1	1.4
Methionine	2.8	2.6	2.7	2.7		3.3	3.5	3	4	3.4
Threonine	6.0	5.1	5.5	5.5		5.4	3.9	2.7	4.1	4.1
Leucine	8.5	6.1	7.4	7.3	7.3*	7.7	12.1	5.8†	24	9.0
Isoleucine	6.2	5.5	5.9	6.0	5.8*	5.2	6.5	3.3†	5	5.3
Valine	5.9	4.6	5.0	5.3	5.7*	5.7	7.0	3.6†	5	6.3

<sup>a</sup> A sample of Food Yeast (*Torula utilis*) gave 6.8% of lysine.

\* Hydrolysis with HI gave results roughly 5% higher.

† Whole Wheat.

Table III summarizes the data in Table II and gives values for the amino acid composition of yeast, meat,<sup>2</sup> casein, white flour, corn and rice.

The results reported in this paper are in general agreement with our earlier studies on a single sample of brewer's yeast (10) and with the

<sup>2</sup> The proportion of collagen to the total protein in lean meat may vary from 3% to 30%.

results of Fink and Just (11) and of Holland, Lyman, and Hale (12). The largest discrepancy is in the case of cystine. It is unlikely that Fink's 1938 cystine value (11) is correct for, in 1943, Hock and Fink (15) reported that the addition of cystine to a yeast diet markedly increases its nutritive value.

Most of the studies on the biological value of yeast proteins were based on feeding yeast as the sole source of dietary protein. These investigations have been reviewed by Carter and Phillips (16) who conclude their study of the literature by stating: "Although these data are obviously inconclusive they indicate that yeast as the sole source of protein may be somewhat inferior to animal proteins in human nutrition." The studies of Hock and Fink (15) and of Klose and Fevold (17) suggest that the primary deficiency in yeast proteins is in the sulfur amino acids, cystine and methionine. Although the former investigators (15) found that cystine markedly improved the nutritive value of yeast, the latter workers (17) could not confirm this finding, but reported that yeast is seriously deficient in only one amino acid, methionine. They also found that yeast, even with added choline, is not as good as casein unless supplemented with methionine.

Although the data in Table III indicate that yeast proteins, like casein, may be mildly deficient in the sulfur amino acids it is rather difficult to explain the findings of Hock and Fink (15) and of Klose and Fevold (17) unless one assumes that the amino acid composition of the yeasts they used differed markedly from each other and from those used in this study. Another possible explanation of their results is that large quantities of non-protein nitrogen (purines, pyrimidines, nucleic acids, etc.) (cf. 18) decreased the availability, or increased the requirements, of cystine and methionine. Examination of Table II, which includes assay results from a large number of different types of commercially available yeasts grown under a variety of environmental conditions, indicates rather remarkable constancy in the cystine-methionine levels.

Practically, however, yeast is not intended to be used as the sole source of protein in any dietary. The recent publicity given to the use of yeast in human foods has been based solely on its supplemental value. On this basis, absolute biological values determined by feeding yeasts to animals as a sole source of protein are of little importance. No one expects to supply all the protein of a ration, whether for human beings or farm animals, in the form of yeast. What is of interest,

especially at the present time, is the value of yeasts in supplementing the amino acid (and vitamin) deficiencies of the cereal grains. Cereal grains are the cheapest, most abundant, most palatable and most widely used source of calories and amino acids. The data in Table III show how yeast proteins are able to improve the nutritive value of cereals, equaling casein in this respect (19, 20).

#### ACKNOWLEDGMENT

We are indebted to Anheuser-Busch, Inc. for a grant which made this investigation possible.

The sulfur analyses were carried out by the Arlington Laboratories.

#### SUMMARY

Eight commercially available yeasts, grown under different conditions, and two extracts prepared from autolyzates have been analyzed for arginine, histidine, lysine, tyrosine, tryptophan, cystine, methionine, threonine, leucine, isoleucine, and valine under highly comparable conditions.

The results indicate that the amino acid content of *Saccharomyces cerevisiae* is quite constant though it may vary somewhat, depending upon the variety and the nutritive medium.

Yeasts are especially valuable food supplements in dietaries where cereal grains furnish all or almost all of the dietary protein.

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## Phenol Conjugation

### III. The Type of Conjugation in Different Species \*

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Received March 20, 1945

#### INTRODUCTION

The fact that phenol conjugates with both sulfuric and glucuronic acids has been known since Baumann (1) and Schmiedeberg (2) discovered both types of conjugation. The purpose of this paper is to investigate the conjugation of phenol in the tissues of various animals *in vitro* and the influence of sulfate upon it.

#### METHODS

The animals were killed by a blow on the head with the exception of frogs which were pithed. The tissues were immediately taken out and sliced according to the Warburg technique. The tissue slices were incubated in 50 ml. Erlenmeyer flasks containing 4 ml. Krebs (3) solution, pH 7.2, phosphate buffer, shaken 100 times a minute. The Krebs solution was modified for use with frog tissues in the following way: 72 ml. 0.9% NaCl, 1.2 ml. 1.15% KCl, 1 ml. 1.221% CaCl<sub>2</sub>, 0.3 ml. 3.82% MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.2 ml. Krebs' phosphate buffer, brought to 100 ml. with distilled water. Phenol concentration, temperature and time of incubation, and weight of tissue per flask are stated in the tables.

The experiments were carried out in air and without addition of glucose. These changes from previous conditions (4) did not affect the results, for conjugation occurs at the same rate whether glucose is present or not and whether the gas phase is air or oxygen.

Total and free phenols were determined according to the Theis and Benedict (5) method and calculated as micrograms in 4 ml. solution.

\* This work has been supported partly by funds provided by the Duke University Research Council.

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TABLE I  
*Influence of Sulfate on Phenol Conjugation by Animal Livers in Vitro*

Medium: Krebs solution, pH = 7.2, phosphate buffer. Gas phase, air. Volume in each flask, 4 ml.

Species	Total phenols	Conjugated phenols	Conjugation coefficient*	Dry weight of tissue	Phenol concentration	t	Condition
Rat	173-188 Av. † 179.5	7-25 Av. 17.4	0.95±0.05(11)	mg. 8-27	mg. in 100 ml.	°C.	With sulfate
	177 Av. 177	4-7 Av. 6	0.36±0.05(3)	15-17	5	37.5	No sulfate
Cat	188-196 Av. 192	4-18 Av. 11	0.67±0.36(2)	17	5	37.5	With sulfate
	186-191 Av. 188.5	4-4 Av. 4	0.23±0.004(2)	17-18	5	37.5	No sulfate
Dog (Puppy)	81.5-89.5 Av. 85.9	7.4-9 Av. 8.5	0.48±0.01(4)	16-19	2.5	37.5	With sulfate
	79-86.5 Av. 82.6	0-3 Av. 1.5	0.09±0.04(4)	16-19	2.5	37.5	No sulfate
	177-195 Av. 185.8	4-17 Av. 10.6	0.61±0.05(12)	16-20	5	37.5	With sulfate
	180-187 Av. 183.5	0-4 Av. 1	0.06±0.02(8)	16-20	5	37.5	No sulfate
Chicken	191-198 Av. 195.2	9-19 Av. 14.5	0.43±0.07(6)	17-19	5	37.5	With sulfate
	193-207 Av. 200.3	10-19 Av. 15.3	0.49±0.06(6)	16-18	5	37.5	No sulfate
Frog ( <i>rana pipiens</i> )	41.5-43.5 Av. 42.8	2.5-3.5 Av. 2.9	0.27±0.02(4)	9-15	1	24.2	With sulfate
	41.5-44 Av. 42.8	0	0 (4)	11-17	1	24.2	No sulfate
Frog ( <i>rana catesbeiana</i> )	43-50.6 Av. 46.6	5.3-9.2 Av. 7	0.43±0.02(8)	16-17	1	20	With sulfate
	44-51.5 Av. 48.2	1.2-7 Av. 4.1	0.25±0.02(8)	15-20	1	20	No sulfate
	214-236 Av. 227.1	1-17 Av. 8	0.51±0.1 (7)	13-17	5	20	With sulfate
	209-221 Av. 215	0-10 Av. 3.6	0.22±0.07(8)	14-17	5	20	No sulfate

\* Micrograms of phenol conjugated per mg. dry weight of tissue in incubation time.

† Av. = average.

Rat and chicken tissues were incubated 1 hour; guinea pig, cat, and dog 90 minutes; frog 2 hours.

The figures in parentheses indicate the number of determinations.

*1. Conjugation in Rats Fed Phenol*

It was previously found (4) that the ability to conjugate phenol increases after phenol feeding. This observation was made on rats which conjugate phenol to a large extent even in the absence of sulfate. In order to determine whether the rats of the Vanderbilt strain (6) behave in the same way, phenol was fed to them.

Rats weighing from 200 to 320 g. were given drinking water containing 3 g. phenol per liter for 9 to 103 days. The rats drank from 30 to 50 ml. a day, which amounts to a total of 0.09 to 0.15 g. of phenol per day.

The results obtained with this strain are quite different from the ones observed with the Rosario Medical School rats (4). There is no increase in the ability to conjugate phenol in the liver and kidney after phenol feeding. This latter tissue and spleen do not appear to conjugate phenol in this strain, whether the animal has or has not been fed phenol.

Phenol feeding had no influence on the conjugation when the liver slices were incubated without sulfate. Magnesium chloride was substituted for the equivalent concentration of magnesium sulfate in these experiments.

*2. Conjugation in Other Species*

Dog (puppies), cat, two species of frog (*rana pipiens* and *rana catesbeiana*) and chicken (New Hampshire Red) liver were studied. Experiments were carried out in the presence and absence of sulfate. The results are shown in the table and from them it may be seen that the livers of all the species studied conjugate phenol. In the absence of sulfate some livers [rat, cat, frog (*rana catesbeiana*)] conjugate to a less extent than in its presence, and in other species [dog, frog (*rana pipiens*)] conjugation does not occur in the absence of this ion.

## DISCUSSION

Evidently the behavior of the rats of the Vanderbilt strain is different from the ones used at Rosario Medical School. Not only is conjugation in the absence of sulfate lower in the first strain, but no increase could be shown in the ability to conjugate phenol in liver and kidney after phenol feeding. Furthermore, spleen does not conjugate phenol whether phenol has been fed or not.

The very low ability for phenol conjugation in absence of sulfate in the Vanderbilt strain indicates the predominance of conjugation with sulfate. The amount of conjugated phenol found by the technique used was not increased after prolonged hydrolysis at 126°C. (30 minutes at 20 pounds pressure), indicating that all conjugated phenol is hydrolyzed after 10 minutes boiling. Therefore, it can safely be stated that this strain conjugates phenol mainly with sulfate. The low conjugation in absence of sulfate can be attributed either to the small amount of sulfate present in the tissue slices or to conjugation with glucuronic acid. In this strain the ability to conjugate phenol does not increase after feeding phenol. On the contrary, in the Rosario Medical School strain previously studied (4), the conjugation coefficient after phenol feeding was higher and more phenol was conjugated in the absence of sulfate. Also the addition of glucuronate restored the conjugation after inhibition by monoiodoacetate. It seems quite clear that the Vanderbilt strain conjugates phenol mainly with sulfate and that the Rosario Medical School rats with both glucuronate and sulfate. If this is the case, phenol administration appears to influence only the conjugation with glucuronate. It has not been possible to obtain direct proof of the presence of glucuronates, after incubation of liver slices with phenol, with Tollens' naphthoresorcinol test. If we consider the values obtained by Lipschitz and Bueding (7) for the formation of phenol glucuronate by guinea pig liver slices (0.4 mg. by 1 g. dry weight of liver, in 90 minutes) it is easy to understand how difficult it will be to detect the formation by 80 to 90 mg. of tissue during one hour incubation.

The very low conjugation in absence of sulfates by other species [dog, frog (*rana pipiens*)] indicates that they probably conjugate phenol mainly as sulfate. Conjugation with glucuronic acid cannot be excluded as it has been discovered by Schmiedeberg (2) and shown to occur by Embden and Glaessner (8) in the dog. This discrepancy between the *in vitro* and *in vivo* results may be explained by the fact that phenol glucuronate is formed in much smaller amounts than phenol sulfate and therefore the former can only be detected when it is concentrated in the urine.

Our results with chicken and frog tissues confirm the *in vivo* observations of Christiani (9).

The interest and helpful criticism of Associate Professor Frederick Bernheim are gratefully acknowledged.

## SUMMARY

1. The ability to conjugate phenol is not increased by phenol feeding in the liver and kidney of the Vanderbilt strain of rats.
2. Kidney and spleen slices do not appear to conjugate phenol in this strain.
3. Livers of dog (puppies), cat, frog, and chicken conjugate phenol.
4. Livers of rat, cat, and frog (*rana catesbeiana*) conjugate phenol to a lesser extent in absence of sulfate.
5. Livers of dog (puppies) and frog (*rana pipiens*) practically do not conjugate phenol in the absence of sulfate.
6. The bearing of these facts on the type of conjugation is discussed.

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# Absorption and Excretion of Calcium Pantothenate by Normal and Depleted Dogs

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Received April 10, 1945

## INTRODUCTION

Although the urinary excretion of pantothenic acid has been studied in a variety of animal species, only one of the investigators attempted to establish a saturation test for the deficiency. Gordon (1942) was unable to detect any difference in the excretion of a test dose of calcium pantothenate by medical students and patients with general nutritional deficiency. Studies made in this laboratory (Silber, 1943) showed that the pantothenic acid level in tissues of depleted dogs could be increased at least 4 mg. per kg. body weight by calcium pantothenate administration, thus indicating the possibility of developing a saturation test for this species. The results of such a study conducted in the course of an investigation of pantothenic acid depletion in dogs (Seeler and Silber, unpublished; Silber, 1943) are reported in this communication. In connection with the standardization of conditions for oral saturation tests, the effect of food upon absorption and excretion of calcium pantothenate has also been studied.

## EXPERIMENTAL

### 1. Saturation Tests

Two adult dogs and seven pups which received the basal diet (Silber, 1943) and B vitamins, exclusive of calcium pantothenate, were used for saturation studies. All except two pups were depleted twice. Three additional pups which were fed a stock diet consisting of dog pellets plus 20 g. dried beef liver daily served as controls. After a period of depletion in which food consumption, growth, and excretion of pantothenic acid were depressed, a dose of 4 mg. calcium pantothenate per kg. body weight was administered, either orally or subcutaneously, following an overnight fast. Food was withheld for 3 to 4 hours after the test dose and 24 hour urines were collected under toluene for pantothenic acid assay with *L. casei* as the test organism

(Silber and Mushett, 1942). The saturation test data are summarized in Tables I and II.

In the 24 hours following oral administration of 4 mg. calcium pantothenate per kg., depleted dogs excreted 0.13%, and normal dogs 1.7% of the test dose. After parenteral administration of the same dose, depleted dogs excreted 3.3%, and their litter mate controls 11.7%. In none of the tests was a significant fraction of the test dose detected in the urine on the second day.

When 10 adult, non-depleted dogs, selected at random, were given 4 mg. calcium pantothenate per kg. orally, an average of 1.4% of the dose was excreted in the urine in 24 hours. Two of these dogs, which showed signs of malnutrition, excreted less than 0.1% of the dose. Three healthy dogs from our stock colony excreted 18, 25, and 27% of a subcutaneous dose of 4 mg. per kg.

TABLE I

*Excretion of an Oral Test Dose of Calcium Pantothenate (4 mg. per kg.)  
by Depleted Dogs and Dogs Fed an Adequate Diet*

Dog No. and sex	Weight kg.	Months of depletion	Urinary P.A., per 24 hours		Per cent of dose found
			Before dose	After dose	
<i>Adults</i>					
103 F	7.7	8	4	50	0.15
103 F	7.7	3	17	115	0.32
113 F	7.0	6	3	15	0.04
113 F	6.2	3	9	23	0.06
<i>Pups</i>					
206 M	4.1	2	8	66	0.35*
213 M	4.2	2	13	20	0.04
280 M	4.2	1	—	3	0.0
296 F	5.4	1	11	110	0.46*
301 M	5.0	1	2	5	0.0
302 M	3.2	1	1	3	0.0
304 M	3.7	1	25	25	0.0
					Average 0.13%
<i>Controls</i>					
282 M	11.5	0	90	500	0.9
299 F	8.1	0	350	850	1.5
303 M	9.5	0	135	1200	2.5
					Average 1.7

\* It is possible that slight contamination from loose stools occurred.

TABLE II

*Excretion of a Subcutaneous Test Dose of Calcium Pantothenate (4 mg. per kg.) by Depleted Dogs and Dogs Fed an Adequate Diet*

Dog No. and sex	Weight kg.	Months of depletion	Urinary P.A., per 24 hours		Per cent of dose found
			Before dose	After dose	
280 M	3.9	1.5	γ	540	3.5
296 F	4.3	1.5	5	640	3.7
301 M	4.1	1.5	2	230	1.4
302 M	3.0	1.5	1	600	5.0
304 M	3.5	1.5	3	420	3.0
					Average 3.3
<i>Controls</i>					—
282 M	9.5	0	60	3300	8.5
299 F	6.4	0	150	3000	11.1
303 M	7.5	0	70	4700	15.4
					Average 11.7

Fourteen to twenty-seven per cent of the administered pantothenic acid was recovered in the 24 hour feces of dogs, whether depleted or normal, when the test dose was given by mouth. However, when it was injected subcutaneously, no increase in fecal pantothenic acid was found.

Evidence of intestinal absorption by the depleted dogs was obtained by determining the urinary excretion of an oral dose of riboflavin. Thus, four pantothenic-acid-depleted pups excreted 8.2% (5.2-11.6) of a 4 mg. per kg. dose of riboflavin whereas 3 control dogs excreted 5.6% (5.0-6.0) of the dose in 24 hours.

That the dogs were actually in a state of pantothenic acid deficiency was borne out by the growth response when calcium pantothenate was supplied daily after the last test dose had been administered. Three pups (No. 280, 296, 302) showed weight increases of 10.2, 7.5, and 7.9 kg., respectively, within 3 months after initiation of calcium pantothenate therapy, whereas in the previous 2½ months they had lost an average of 0.8 kg. each. Similar weight responses resulted when two depleted adult dogs received daily oral therapy. When the 4 mg. per kg. dose was administered daily, the urinary excretion of pantothenic acid gradually increased from 0.2% to 20-30% of the dose within 2 weeks.

In order to obtain information regarding the excretion of oral doses of calcium pantothenate by men, tests were performed on a group of 10 laboratory workers. Since the peak excretion of pantothenic acid after oral administration occurs within the first 4 hours, this was employed as the test period. Two and one half hours after

a light breakfast, the bladder was emptied and 50 mg. of calcium pantothenate were given with 200 cc. of water. Four hour urines were assayed for their pantothenic acid content along with control urines collected in the same manner the previous day. Prior to dosing,  $1.0 \pm 0.15^*$  mg. pantothenic acid was excreted in the 4 hour period; after the test dose, this figure was raised to  $6.0 \pm 0.48$  mg.\*

## *2. Effect of Food on Absorption and Excretion of Vitamins*

In a study of riboflavin and pantothenic acid deficiency in dogs, it was found that as food consumption was depressed in the advanced stages of riboflavin deficiency, the excretion of orally administered calcium pantothenate increased. Furthermore, when pantothenic acid deficiency was induced, again with a decrease in food consumption, the excretion of orally administered riboflavin increased. These results suggested a relationship between the two vitamins similar to that observed by Sure and Ford (1942) for thiamine and riboflavin in thiamine-depleted rats. However, on analysis, the effect was seen to be solely a result of enhanced absorption from the intestinal tract in the absence of food, as demonstrated in the following experiments.

Four dogs which for 6 months had been receiving the basal diet plus a daily supplement of B vitamins (including 40 mg. calcium pantothenate and 1 mg. riboflavin) were given their vitamins in the morning with graded amounts (0–100 g.) of basal diet. Additional diet was supplied *ad libitum* 4 hours later and 24 hour urine and feces samples were assayed for the vitamins. The urinary excretion of both vitamins increased when no food was given with the vitamins and decreased when food was supplied (Table III). Fecal assays showed that the decrease in urinary excretion of pantothenic acid could be correlated with an increase in the fraction of the dose which passed through the intestinal tract unabsorbed. Excretion of pantothenic acid into the intestinal tract did not appear to be involved since parenterally administered calcium pantothenate was not excreted in the feces.

Furthermore, when two dogs had their food intake restricted to 15 g. per day for one week and the B vitamins were administered orally as above, 35 to 65% of the 40 mg. dose of calcium pantothenate was excreted in the urine in 24 hours. When food was then supplied *ad libitum* with the vitamin supplement, the urinary excretion decreased rapidly until one month later it was only 1 to 2.5% of the dose. The food consumption at this time averaged 370 g. per day.

Thus, when the food intake was decreased (either by a vitamin deficiency or by restricting food intake), the urinary excretion of calcium pantothenate was increased by as much as 50% of the amount fed as a dietary supplement and when food was supplied with the vitamins, as much as 50% passed through the intestinal tract unabsorbed.

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\* Standard Error.

TABLE III

*Influence of Food Consumption on the 24 Hour Excretion of Pantothenic Acid (40 mg.) and Riboflavin (1 mg.)*

Dog	Diet given with the vitamins	Total diet consumed	Urinary excretion		Fecal excretion	
			P.A.	Ribo.	P.A.	Ribo.
No.	g.	g.		mg.		mg.
202	0	140	7.2	0.45	2.0	0.05
203	0	135	1.7	0.86	10.5	0.13
204	0	220	9.2	0.70	9.5	0.12
205	0	65	4.7	0.84	—	—
Averages			5.7	0.7	7.3	0.10
202	25	155	2.8	0.45	23.0	0.27
203	25	225	2.0	0.40	11.0	0.18
204	25	145	5.5	0.37	11.5	0.25
205	25	135	4.5	0.45	7.5	0.10
Averages			3.7	0.42	13.0	0.25
202	50	130	2.4	0.27	11.0	0.13
203	50	135	0.25	0.08	12.5	0.18
204	50	150	1.25	0.45	15.0	0.14
205	50	225	4.7	0.52	7.0	0.08
Averages			2.15	0.33	11.4	0.13
202	100	210	0.6	0.30	—	—
203	100	240	0.15	0.05	22.0	0.34
204	100	130	0.70	0.60	12.5	0.14
205	100	135	2.0	0.35	19.0	0.25
Averages			0.86	0.30	17.8	0.18

## DISCUSSION

Gordon (1942), in his study conducted with medical students and patients, did not find any significant difference in the excretion of test doses (10 or 20 mg.) of calcium pantothenate. In our experiments, however, which were conducted on severely depleted dogs, a marked difference was found between the controls and the pantothenic-acid-deficient animals. Normal dogs excreted 13 times as much of an oral

dose of 4 mg. calcium pantothenate per kg. as depleted dogs. After a similar dose was administered subcutaneously, more of the vitamin appeared in the urines of both groups, and the control dogs excreted only 3.5 times as much as the depleted animals. In terms of total urinary excretion, after oral administration, normal dogs excreted 1.6% more of the dose than depleted dogs; and after subcutaneous administration, excretion by the normal dogs exceeded that of the depleted dogs by 8.5% of the dose. Faulty intestinal absorption did not appear to be a complicating factor in the oral test because the pantothenic-acid-depleted dogs excreted as much of a test dose of riboflavin as the control dogs.

An increased urinary excretion of riboflavin as a result of thiamine deficiency (Sure and Ford, 1942) or as a result of thiamine administration (Klopp, Abels, and Rhoads, 1943) has been interpreted as evidence of a vitamin interrelationship. It has also been reported that in thiamine-deficient rats the riboflavin content of tissues decreases (Sure and Ford, 1942) and the riboflavin level of the liver increases (Singher, *et al.*, 1944), that thiamine or pantothenic-acid-depleted rats are limited in their ability to mobilize riboflavin in the liver after feeding (Supplee, *et al.*, 1942), and that pantothenic acid and riboflavin blood levels in man are related because an increase in one was accompanied by an increase in the other (Spies, *et al.*, 1940). Ferrebee and Weissman (1943) also found lower levels of riboflavin in the livers of rats depleted of thiamine. They felt, however, that it was not significant because the levels of riboflavin were essentially the same throughout the depletion period: They also regarded the increased excretion of riboflavin in terminal thiamine deficiency as a non-specific effect due to tissue catabolism. In a 2 hour excretion experiment in dogs (Silber and Unna, 1942), no effect of the intravenous administration of calcium pantothenate on the riboflavin levels in the blood or urine was observed and similarly, when sodium riboflavinate was administered, no effect on the pantothenic acid levels was found.

The present experiments indicate that when excretion data are used to evaluate the status of a deficiency or to demonstrate vitamin interrelationships, extreme care must be taken to make valid comparisons. Not only must food intake be controlled but the vitamin supplement must be administered to the test animals and their controls in exactly the same manner, preferably orally after overnight fasting or parenterally, so that the presence of food in the stomach will not

confuse the test. Since the administration of pantothenic acid with food may result in the loss of a large fraction in the feces, it appears likely that tissue levels may also be higher or lower depending upon the way in which the vitamin supplement is supplied.

### SUMMARY

(1) Significant differences in the excretion of oral or subcutaneous test doses of calcium pantothenate by normal and depleted dogs have been found. Intestinal absorption did not appear to be impaired by pantothenic acid depletion as judged by excretion of oral doses of riboflavin.

(2) The urinary excretion of an oral dose of calcium pantothenate by dogs may be as much as 50% or as little as 1% of the dose, in inverse relation to the amount of diet consumed with the vitamin. When food was supplied *ad libitum*, a large fraction of the dose passed through the intestinal tract unabsorbed. It is suggested that this factor be considered in experiments designed to demonstrate vitamin interrelationships on the basis of vitamin levels.

(3) After parenteral administration of calcium pantothenate, no increase in fecal pantothenic acid was detected.

(4) In a four hour period, a group of 10 laboratory workers excreted 10% of a 50 mg. dose of calcium pantothenate.

### ACKNOWLEDGMENT

The author wishes to express his appreciation to Miss Dorothy Ferguson, Mr. John Flanagan, and Mr. Joseph O'Leary for valuable technical assistance.

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# **Alterations in Particulate Glycogen Produced by the Common Glycogen Separating Agents**

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Received April 12, 1945

## **INTRODUCTION**

In a previous publication (1) the separation of particulate glycogen from the liver of the guinea pig was described. Particulate glycogen is one of several submicroscopic components within the cytoplasm of the liver cell (2). It is visible as a particle under the ultramicroscope. It is separated from the cell by mechanical rupture of the cytoplasmic membranes and differential centrifugation of the resulting liver emulsion. By repeated centrifugations a highly purified glycogen preparation containing 0.17% nitrogen and 0.019% phosphorus was obtained. Solutions of particulate glycogen show marked opalescence, and the glycogen can be almost completely removed from solution by thirty minutes of centrifugation at 12,000 g.

Inasmuch as this type of glycogen had not previously been obtained, it was deemed advisable to test the effect on particulate glycogen of the common agents used in the chemical separation of glycogen. It was found that these agents, *i.e.*, heat, trichloroacetic acid, and potassium hydroxide, were not without effect on particulate glycogen. All of these agents produced a permanent dispersion of the particle—that is, they produced a marked reduction in the opalescence of the solution; the glycogen could no longer be completely removed from solution at 12,000 g. in thirty minutes, and the particulate character as seen in the ultramicroscope was lost. This dispersed glycogen has some residual opalescence and can be removed from solution by ultracentrifugation, although higher gravitational fields and much longer times are required. The other properties of glycogen, *i.e.*, iodine reaction and precipitability by alcohol, are only affected slightly, if at all.

### EXPERIMENTAL

A preparation of four times washed particulate glycogen was prepared as previously described (1). It was stored as a 1% solution at -20°C. until ready for use.

#### *Opalescence*

Fig. 1 shows the opalescence of particulate glycogen solutions plotted against the concentration. The opalescence was measured in an Evelyn colorimeter using a 420 filter and an appropriate blank.

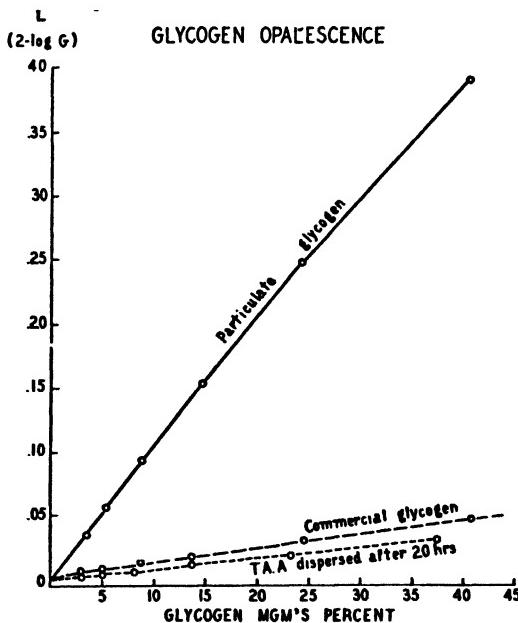


FIG. 1

It is to be noted that the photometric density ( $2 - \log G$ ) plotted against the concentration of glycogen is a fairly straight line. Particulate glycogen dispersed by 4% trichloroacetic acid shows a marked reduction in opalescence. For comparison the opalescence of commercial glycogen is shown. In spite of this marked decrease in opalescence there is no change in glycogen content on chemical analysis (alcohol precipitation, hydrolysis, and glucose determination).

### *Dispersion Rate*

Fig. 2 shows the rates of particulate glycogen dispersion plotted against time for various agents. With 4% trichloroacetic acid the initial dispersion is very rapid, the opalescence decreasing by 60% within the first minute. After one and a half hours the opalescence

RATE OF GLYCOGEN DISPERSION

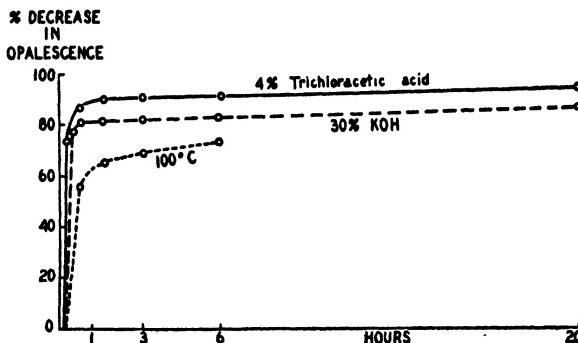


FIG. 2

decreases by 90% and it falls to only 6% of its original value in 21 hours. Although 30% KOH produces an initial rapid dispersion, this is apparently not quite as complete as that produced by the trichloroacetic acid, for even after 21 hours it has lost only 84% of its opalescence. Heating at 100°C. disperses the glycogen at a somewhat slower rate than KOH or trichloroacetic acid.

### *Glycogen-Iodine Complex*

In order to measure the glycogen-iodine color complex in the Evelyn photoelectric colorimeter, a 600 m $\mu$  filter was used. At this wave length the absorption due to the iodine solution alone or to the opalescence of the glycogen solution alone is small compared to that of the glycogen-iodine complex. In order to compare the iodine color complex of particulate and dispersed glycogens it is necessary to correct for the difference in opalescence of particulate and dispersed glycogens. The transmissions (% of light transmitted) of the following solutions were measured against a water blank--(1) Iodine blank ( $T_I$ ), (2) Glycogen blank ( $T_G$ ), (3) Glycogen-iodine complex ( $T_{GI}$ ). The value  $T$  given in the equation represents the change in transmission due to the formation of the glycogen-iodine complex and would represent the transmission

$$T = \frac{T_{GI}}{T_G \times T_I}$$

as compared with a blank containing uncombined iodine and glycogen.

The dispersed glycogen sample was prepared by treatment with 4% trichloroacetic acid for thirty minutes, after which time the acid was carefully neutralized with NaOH and phosphate buffer to pH 6.8. To the particulate glycogen sample an equivalent amount of neutralized trichloroacetic acid and phosphate buffer at pH 6.8 were added. The absorptions are given in Table I.

TABLE I  
*Glycogen-Iodine Complex Absorption at 600 m $\mu$*

	Particulate glycogen	Dispersed glycogen
Concentration of glycogen mg. %	20 mg. %	20 mg. %
Concentration of iodine in KI approx.	.001 N	.001 N
T <sub>I</sub> (% Transmission iodine alone)	96%	96%
T <sub>G</sub> (% Transmission glycogen alone)	87.7%	98%
T <sub>GI</sub> (% Transmission glycogen-iodine complex)	43.5%	55.3%
T Calculated transmission corrected for blanks	51.7%	58.7%

Thus, in spite of a marked decrease in opalescence in the particulate glycogen upon dispersion with trichloroacetic acid, the glycogen-iodine complex changes a relatively small extent.

#### *Effect of Alcohol Precipitation*

Particulate glycogen solutions precipitated by two volumes of 95% alcohol and resuspended in the original volume of water showed no change in opalescence. Drying of the precipitated cake for 1 hour *in vacuo* over P<sub>2</sub>O<sub>5</sub> produced no alteration. Even heating while in the dry state produced no change in opalescence upon resuspending in water. This is unlike the effect of heating particulate glycogen while it is suspended in water.

TABLE II  
*Opalescence of Particulate Glycogen Solutions after Various Treatments and Resuspension in the Original Volume*

	Incident light transmitted I/I <sub>0</sub> per cent
No treatment	67.7
Alcohol precipitation	66.7
Alcohol precipitation and dried	67.3
Alcohol precipitation, dried, heated 100°C. 20 min.	67.7

Although preparations of particulate glycogen precipitated by alcohol and dried disperse fairly readily in water, air-dried particulate glycogen preparations which have been extracted with alcohol and

ether and stored for several months show a decreased tendency to disperse in water.

### *Centrifugability*

Table III shows the change in centrifugability of glycogen after dispersion. Samples of particulate glycogen were dispersed at 5°C. for 3 and 2½ hours respectively in 3% trichloroacetic acid and 30% KOH. The glycogen solutions were centrifuged

TABLE III  
*Glycogen Centrifugation in Water*  
Original Glycogen Concentration = 20 mg. %

Gravitational field	Time of centrifugation minutes	Particulate per cent	Glycogen removed on centrifugation Trichloroacetic acid dispersed per cent	KOH dispersed per cent
1,600	30	61	5	18
6,500	30	76	18	28
14,600	30	97	45	50
23,000	30	99	47	52
23,000	93	—	55	68
23,000	180	—	74	76

in the multispeed attachment for the International centrifuge at various gravitational fields and for various times. An aliquot of the supernatant was removed with a pipette, and after hydrolysis in 5 N H<sub>2</sub>SO<sub>4</sub> for 20 minutes the glycogen was determined as glucose by the Folin micro method (3), and the color absorbed was measured in an Evelyn colorimeter. The percentage of glycogen removed is given in Table III. After centrifugation at 1,600 g. for 30 minutes more than 60% of the particulate

### RATE OF GLYCOGEN SEDIMENTATION

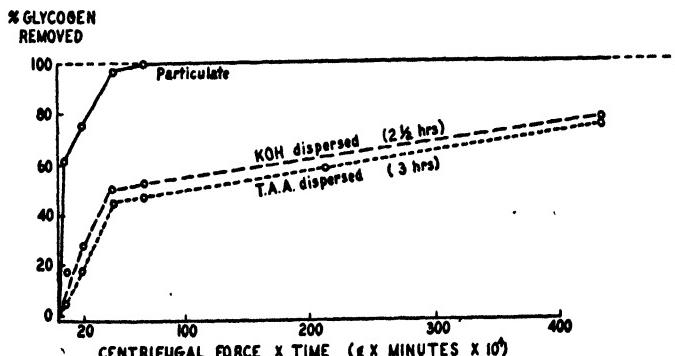


Fig. 3

glycogen was removed, whereas less than 5% of the trichloroacetic acid dispersed was removed. Ninety-nine per cent of the particulate glycogen was removed in 30 minutes centrifugation at 23,000 g., whereas only 47% of the trichloroacetic dispersed glycogen was removed in the same time.

Fig. 3 is a plot correlating the percentage of glycogen removed to the force and time of centrifugation expressed as gravitational force times minutes. The trichloroacetic acid and KOH dispersed glycogens appear to show two slopes. The initial slope seems to simulate that of particulate glycogen. Since Fig. 2 shows that there is progressive glycogen dispersion with time, and since these dispersions were carried out at 5°C. (the dispersions shown in Fig. 2 were carried out at 25°C.), this initial slope is probably due to remaining particulate glycogen. The final slope is very gradual and presumably represents the sedimentation of dispersed glycogen. At this rate it would take over 5 hours to completely remove the dispersed glycogen at 23,000 g.

#### *Permanence of Trichloroacetic Acid and KOH Dispersion*

Attempts at reformation of particulate glycogen were unsuccessful. Particulate glycogen was dispersed with 5% trichloroacetic acid and 30% KOH. After allowing 10 minutes for dispersion the dispersing solution was carefully neutralized, buffer solutions of various hydrogen ion concentrations from pH 3.6 to 10.0 were added, and the glycogen allowed to stand. The opalescence was read at intervals up to 15 hours in an Evelyn colorimeter. There was no change in opalescence and hence no evidence of particulate glycogen reformation.

#### DISCUSSION

It has been suggested (1) that the very small amount of nitrogen present in particulate glycogen, which is, in part at least, due to protein, might be an important factor in holding the particle together inasmuch as the three agents which disperse particulate glycogen markedly alter proteins but do not affect ordinary glycogen. Furthermore, combinations of glycogen with protein are known to occur (4, 5). However, electrophoretic studies on particulate glycogen have not yet been carried out so that we do not have any direct evidence of the protein complex.

It is apparent, however, that, in studying the factors involved in the glycogen breakdown and synthesis within the liver cell, one must consider the physical states of the glycogen as it occurs within the living cell.

Loring and Pierce (6) have recently separated rabbit liver glycogen by ultracentrifugation at 79,000 g. for 2 hours. However, they used a 3% trichloroacetic extract which, as we have shown, dispersed the particulate glycogen. Their glycogen, therefore, corresponds to our dispersed glycogen. They report .0015% phosphorus and nitrogen less than the experimental error of a micro-Kjeldahl. Although these values suggest a slightly purer preparation of glycogen than was first reported (1), the lower nitrogen and phosphorus values might also be the secondary result of dispersion of the "native" glycogen of the cell by trichloroacetic acid and removal of protein.

### SUMMARY

1. Particulate glycogen represents the native state of glycogen in the liver cell.
2. It is dispersed by the common agents used in the chemical separation of glycogen from the cell, i.e., heat, 4% trichloroacetic acid, and 30% KOH.
3. Particulate glycogen is not altered by precipitation with alcohol and it may be heated in the dry state without effect.
4. Ninety-nine per cent of the particulate glycogen is removed by centrifugation at 23,000 g. for thirty minutes.
5. Glycogen dispersed by trichloroacetic acid and KOH is slowly removed at 23,000 g.
6. The nature of the bond responsible for particulate glycogen has not been determined as yet.

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# **Studies on the Strength of Fibrinogen-Thrombin Clots \***

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Received April 19, 1945

## **INTRODUCTION**

Fibrin clots have been put to increasing use within the past few years. They were introduced for the purpose of suturing nerves both in the experimental animal (1, 2, 3) and in man (4, 5, 6), and they have since been used for the repair of wounds of the skin and liver as well as for the fixation of skin grafts (7, 8, 9), and for the removal of renal calculi (10). Fibrin films (11) have been found useful in the treatment of surface burns (12, 13), and in the repair of dural defects and the prevention of meningocephalic adhesions (14) while fibrin foam is being employed as a hemostatic agent (15). As clot strength appears to be of some importance in relation to some of these usages, it has seemed worthwhile to investigate clots prepared from fibrinogen and thrombin from the standpoint of attempting to obtain as strong a clot as possible. While our previous investigations have been concerned with some physical properties of various types of plasma clots (16, 17, 18, 19), this study deals mainly with the effect of various divalent ions on the breaking strength of clots prepared from human fibrinogen (20) and human thrombin.† The fibrinogen preparations contained a considerable amount of nonclotting protein and 30–35% of sodium citrate.

\* The work described in this paper was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the Jewish Hospital of Brooklyn, New York.

† The products of plasma fractionation employed in this work were made from blood collected by the American Red Cross by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under contract, recommended by the Committee on Medical Research, between the Office of Scientific Development and Harvard University.

### EXPERIMENTAL

The method previously described (18) for testing the strength \* of plasma clots was found unsuitable for fibrinogen-thrombin clots. The latter appeared to be more brittle than plasma clots, and the application of the radio clamp, alone or in combination with a small weight load, was occasionally found to result in breakage of the clots at the point of application of the clamp. Therefore, the Neurath, Dees, and Fox (21) modification of incorporating pledgets of gauze at the ends of the clot was adopted. The gauze at the lower end of the coagulum was secured in a second radio clamp, and a double burette system with an attached "S" shaped outlet tube was used to deliver a steady flow of water at the rate of approximately one gram per second. All clots were placed in a water thermostat at  $37 \pm 0.05^{\circ}\text{C}$ . as soon as prepared. Clot strength measurements were made 60 minutes after the "tip test" was positive, since it has been shown (22) that after this time there is hardly any change in the breakage strength. Each clot strength is the average of six determinations unless otherwise indicated. Determinations of pH were made with a glass electrode assembly (Beckman pH meter).

#### *Effect of Calcium Chloride on Clot Strength*

Table I shows the result of using solutions of different quantities of calcium chloride on three solutions containing different percentages of fibrinogen.

TABLE I  
*Effect of Calcium Chloride on Clot Strength (in grams)*

% Fibrinogen	% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and pH of solution															
	0	pH	5	pH	10	pH	15	pH	20	pH	30	pH	40	pH	50	pH
2.3	36	6.10	91	6.08	88	6.01	83	5.91	94	5.89	64	5.68	67	5.60	22	5.37
3.3	37	6.05	107	6.00	151	5.95	152	5.92	130	5.89	114	5.71	87	5.70	99	5.57
4.4	37	6.05	159	6.04	126	6.02	159	5.99	172	5.95	125	5.84	135	5.80	139	5.84

A typical experiment for this determination may be described as follows: 1.320 g. of the fibrinogen preparation are dissolved in 11.3 cc. of distilled water. To this solution is added 0.3 cc. of freshly prepared  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  of the desired concentration. Then 1.6 cc. of thrombin solution containing approximately 70 units of enzyme is added. The amount of thrombin used in each experiment was that quantity necessary to give maximum clot strength of the particular fibrinogen preparation used. From this clotting mixture, six 1.65 cc. portions are immediately transferred to cylindrical glass tubes and incubated in the thermostat at  $37^{\circ}\text{C}$ . The remaining portion is used

\* The term "breaking strength" (or "tensile strength") as used in this investigation refers merely to the load at which the clot breaks under the specified conditions of preparation and testing.

for a pH measurement. The controls contain the same quantity of fibrinogen and thrombin and the same total volume of solution but no calcium chloride. The figures given in Table I are the result of 144 individual tests of breakage strength.

It is evident that the use of  $\text{CaCl}_2$  results in a maximum strengthening effect on the fibrin clots as follows: 2.6 times for the 2.3% fibrinogen preparation; 4.1 times for the 3.3% fibrinogen solution, and 4.6 times for the solution containing 4.4% fibrinogen. In each case in Table I, there is a range of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  percentage, giving an optimum strengthening effect which for the 2.3% fibrinogen is approximately 5–20%; for the 3.3 and 4.4% fibrinogen is roughly 5–30%.

In each fibrinogen solution, there was a small change in pH upon the addition of increasing quantities of calcium chloride (Table I). The effect of changes of pH within the range 5.4 to 6.1 upon the clot strength was studied in order to determine whether the increase in clot strength is due to pH changes which occur upon the addition of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ .

In the following experiments, using 3.3% fibrinogen preparations, varying pH values were obtained by the addition of different quantities of 0.1 N HCl. As in the previous cases, the same quantity of thrombin and total volume of solution were used. For comparison, in three experiments pH values of 6.8, 7.1, and 8.3 were obtained by using different quantities of 1%  $\text{NH}_3$ . The results are given in Table II.

TABLE II  
*Effect of pH on Clot Strength (in grams) of Fibrin Clots*

pH	5.0	5.7	5.8	5.9	6.1	6.8	7.1	8.3
Clot strength	0	20	32	32	37	62	73	0

These results are similar to those obtained by other investigators (21) in that clots become weaker as the pH decreased from 6.1 to 5.0. It follows, therefore, that the strengthening effect of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  upon fibrinogen thrombin clots cannot be ascribed to pH changes resulting from the addition of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . As a matter of fact, these pH alterations tend to weaken the clots.

It was also decided to test the effect of other calcium salts on the strength of fibrinogen clots. Using a 1.3% fibrinogen solution, a control (value) of 30 g. (in the absence of any calcium compound) at a pH of 6.3 was obtained. Under similar conditions, but in the presence of

10% solutions of the following salts, the results were as follows: With  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 80 g.;  $\text{Ca}(\text{NO}_3)_2$ , 78 g.;  $\text{CaBr}_2$ , 70 g.;  $\text{Ca}(\text{C}_2\text{H}_3\text{O}_2)_2$ , 61 g. The conclusion follows that adequate quantities of  $\text{Ca}^{++}$  increase the tensile strength of clots prepared from fibrinogen solutions containing sodium citrate.

It might be mentioned here that clots made from preparations containing added  $\text{Ca}^{++}$  were milkier (more opaque) than clots made from preparations to which no  $\text{Ca}^{++}$  was added.

### *The Effect of $\text{CaCl}_2$ upon the Stretching of Clots*

In carrying out the many experiments described, it was noticed that not only did the addition of calcium chloride increase the clot strength, but it reduced the clot stretch. In general, the fibrin clots prepared without calcium chloride showed an increase in stretch of 5–6 times as compared with clots prepared with calcium chloride. Also, clots made with the fibrinogen preparations used in this study and containing optimum amounts of calcium chloride stretched uniformly with minimal necking of the clot, while fibrin clots containing no  $\text{CaCl}_2$  showed considerable reduction in the diameter of the mid-portion of the clot.

A series of experiments showing how  $\text{CaCl}_2$  influences the clot stretch for a 2.3% fibrinogen preparation at pH 6.0 is shown in the accompanying Table III. The length of all the clots before measurement was 3 cm.

TABLE III  
*Effect of  $\text{CaCl}_2$  on Clot Stretch of a 2.3% Fibrinogen Solution*

% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0	5	7.5	10	15	20	30	50
pH	6.11	6.08	6.07	6.01	5.91	5.89	5.68	5.37
Increase in stretch in cm.	9	1.5	1.5	1.5	1.7	1.8	1.7	1.5

Reduction of the degree of clot stretch was also obtained with the other concentrations of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . In addition, it was observed that the reduction in clot stretch was generally greater around pH 5.6–6.0 than around pH 7.0.

### *Influence of $\text{Mg}^{++}$ , $\text{Ca}^{++}$ , $\text{Sr}^{++}$ , and $\text{Ba}^{++}$ on the Clot Strength*

It seemed of interest at this point to compare the effect of several other divalent ions on the strength of fibrin clots. The experiments

show that whereas  $\text{Ca}^{++}$  strengthens the clots,  $\text{Mg}^{++}$  and  $\text{Ba}^{++}$  have no such effect at all; while  $\text{Sr}^{++}$  gives a very slight strengthening effect.

### *Strengthening Effect of $\text{CaCl}_2$ at pH 6.9-7.5*

The optimum pH for the clot strength of fibrin has been determined by Neurath, Dees, and Fox (21) to be at 7.2. For our experiments, the pH values were adjusted to the neighborhood of 7.0 by use of an appropriate quantity of 1%  $\text{NH}_3$ . As demonstrated in Table IV, the strengthening effect of  $\text{Ca}^{++}$  is also obtained at pH 6.9-7.2.

TABLE IV  
*Strengthening of Fibrin Clots, Using  $\text{CaCl}_2$  at pH 6.9-7.5*

% Fibrinogen	% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	pH
2.3	0	10
	27	94
	33	78
% Fibrinogen	% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	pH
3.3	0	15
	69	143
	73	187
% Fibrinogen	% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	pH
4.4	0	10
	28	133
	40	124

### *Use of Other Preparations of Fibrinogen (Fraction I (20))*

Each of the five preparations of fibrinogen (20) supplied to us over a period of a year showed an increase of clot strength upon the addition of  $\text{CaCl}_2$ . Table V represents only one of a series of tables prepared from data obtained with fibrinogen preparation No. 5 with the addition of smaller increments of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  than previously reported. The following modifications were also employed. Each 4 cc. of 3.3% fibrinogen solution contained 400 mg. of dried material of Fraction I, 0.1 cc. of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  of the percentage listed, and 0.5 cc. of thrombin solution containing 18 units. In these experiments, 1 cc. of the clotting mixture instead of the usual 1.65 cc. was used for testing. Six to eight clots were tested for each clot strength listed.

TABLE V

*Effect of CaCl<sub>2</sub> on 3.3% Fibrinogen No. V at pH 6.0*

% CaCl <sub>2</sub> ·2H <sub>2</sub> O	0	2.5	5	7.5	10	12.5	15	17.5	20	25	30	40
Clot strength (in grams)	42	171	211	238	247	219	211	200	189	180	176	169

Under these conditions, the strongest clot obtained was 5.9 times stronger than the control.

#### *Strength of Fibrin Clots Containing CaCl<sub>2</sub> Compared with Unmodified Plasma Clots*

It is interesting to compare the clot strength of fibrinogen clots containing CaCl<sub>2</sub> with that of plasma clots. In one study, the average clot strength of eight healthy adult individuals was found to average 44 g. (16), and in another, the figure for eleven normal adults was 46 g. (17). In these experiments, the method described in (16) was used to measure the clot strength. Employing this method and working with 3.3% fibrinogen preparations obtained from other samples of this material, many experiments were carried out. The controls for two such experiments were 26 and 45 at pH 5.9, while the clot strengths in the presence of 0.1 cc. of 8% CaCl<sub>2</sub>·2H<sub>2</sub>O per 4 cc. of clotting mixture were 219 and 203, respectively.

Therefore, regardless of the method used for testing clot strength or the fibrinogen preparation used, it was found that suitable amounts of calcium chloride strengthened clots prepared from fibrinogen-thrombin mixtures containing about 30–35% sodium citrate. In addition, clots prepared from fibrinogen and thrombin are stronger than plasma clots whether the plasma is native or reconstituted from dehydrated preparations of unmodified citrated or oxalated plasma (19).

#### DISCUSSION

For purposes of nerve suture, clots prepared from fresh plasma appear to be preferable to fibrinogen-thrombin clots since the former are more readily absorbed, leaving a smooth epineurial sheath. However, in view of the fact that the physical properties of fibrinogen-thrombin clots may be altered by appropriate measures (23), it is

possible that these strengthened clots may in the future prove adaptable to nerve repair. At any rate, the information obtained in this study on the strengthening effect of calcium chloride upon fibrinogen-thrombin clots may prove helpful in relation to other uses for these materials.

Thanks are due to Dr. A. E. Sobel for his helpful interest in the work. We are pleased to acknowledge the technical assistance of Philip Kutner and Sam I. Miller.

### CONCLUSIONS AND SUMMARY

1.  $\text{CaCl}_2$  strengthens clots prepared from fibrinogen-thrombin preparations containing considerable quantities of sodium citrate; and the addition of calcium ions also reduces the clot stretch.
2. This strengthening effect can be obtained in the neighborhood of the optimum pH for clot strength (7.2) as well as at pH 5.5–6.0.
3. This effect cannot be attributed to the slight changes in pH on addition of  $\text{CaCl}_2$  to the fibrinogen-thrombin mixtures.
4.  $\text{Mg}^{++}$  and  $\text{Ba}^{++}$  do not affect the clot strength while  $\text{Sr}^{++}$  exerts a slight positive effect.
5. Clots prepared from fibrinogen-thrombin mixtures containing added  $\text{CaCl}_2$  are considerably stronger than plasma clots whether freshly prepared or reconstituted from dehydrated plasma.

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# The Effect of the Pantothenic Acid Content of Eggs on the Amount in Newly Hatched Chicks

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Received April 28, 1945

## INTRODUCTION

The effect of incubation on the amounts of pantothenic acid, nicotinic acid, riboflavin, biotin, and inositol in the eggs of hens has been studied by Snell and Quarles (1941). Westenbrink and Van Leer (1941) reported that a considerable part of the thiamine of the eggs is lost during embryonic development of the chick. More recently Serimshaw, *et al.* (1944) reported that the thiamine content of eggs does not change during incubation.

The previous studies on the effect of embryonic development on the amounts of the various vitamins in the egg were made without any particular attention to the amounts of the vitamins in the diet of the hens. Snell, *et al.* (1941) showed that the amount of pantothenic acid in the egg is dependent upon the level in the diet. Likewise, it has been well established that a similar relationship exists for riboflavin, and it would not be surprising if such a relationship also occurs with other B vitamins essential for the chick. It is possible that the amount of vitamin in the egg might be a factor influencing changes in the vitamin content during incubation. This might also be involved in the conflicting results of Westenbrink and Van Leer (1941) and those reported by Serimshaw, *et al.* (1944).

In the present study, hens were fed rations containing different amounts of pantothenic acid. Some of the eggs from these hens were assayed for pantothenic acid and others were incubated and the amount of pantothenic acid in the entire chick at the time of hatching determined.

### EXPERIMENTAL

The basal ration had the following percentage composition by weight: ground yellow corn 54, dried beet pulp 20, meat and bone scraps 5, fish meal 3, corn gluten meal 12, steamed bone meal 2, oyster shell 2, fish oil 0.5, and sodium chloride 0.5. Synthetic riboflavin was added at the rate of 230  $\mu\text{g}$ . per 100 g. of ration. Samples of the mixed ration, collected at intervals during the course of the experiment, gave an average pantothenic acid content of 387  $\mu\text{g}$ . per 100 g. *d*-Calcium pantothenate was added to the basal ration at levels such that ration II contained 857  $\mu\text{g}$ . of pantothenic acid per 100 g. and ration III 1574  $\mu\text{g}$ . per 100 g. Since the amount of pantothenic acid in the egg is directly proportional to that of the diet (Snell, *et al.*, 1941) it was anticipated that hens fed rations I, II, and III would produce eggs containing quite different amounts of pantothenic acid.

The hens used in these experiments were chiefly White Leghorns. They had been fed the experimental rations until the pantothenic acid content of the eggs of each group reached a fairly constant level. During the course of the experiment, some of the eggs were assayed for pantothenic acid, while others were used for incubation purposes.

Pantothenic acid was determined by the microbiological method described by Neal and Strong (1943). The egg was broken out of the shell, weighed and then mixed with water in a Waring blender. An aliquot of suitable size was then added to a 1% solution of sodium acetate, adjusted with acetic acid to a pH of 4.6. The sample was then autoclaved for 30 minutes and filtered. Preliminary trials showed that no additional pantothenic acid is liberated from eggs by enzymatic treatment. The newly hatched chicks were killed without loss of blood and then frozen. The down was burned off over an open flame. The legs and beaks were cut off and the chicks then weighed. Each chick was cut into small pieces and then minced in a Waring blender in water. It was then autoclaved at 15 pounds pressure for 30 minutes, and again put in the blender for a few minutes. This treatment produced a homogenous suspension from which an aliquot was taken with a pipette. This was incubated with both papain and takadiastase for the liberation of pantothenic acid.

### RESULTS AND DISCUSSION

The results of feeding different levels of pantothenic acid to hens, and its relationship to the amounts in eggs and newly hatched chicks, are summarized in Table I. The individual values have been omitted,

TABLE I  
*The Pantothenic Acid Content of Eggs and Chicks*  
 (Values in micrograms per gram)

Pantothenic acid/g. of hen ration	Group I		Group II		Group III	
	Eggs	Chicks	Eggs	Chicks	Eggs	Chicks
Mean and standard deviation for 25 observations	4.9 $\pm$ 0.9	5.2 $\pm$ 1.0	7.9 $\pm$ 1.0	8.3 $\pm$ 1.4	14.0 $\pm$ 1.7	13.6 $\pm$ 1.8

to conserve space, and the data treated statistically. The degree of variability within each group is indicated by the magnitude of the standard deviation.

The amount of pantothenic acid in the egg is definitely dependent on the amount in the ration fed the hens. Within each group there was no significant difference (based on Fisher's *t* value) between the pantothenic acid content of the eggs and the amount in newly hatched chicks. Thus, it is apparent that there is no significant change in the pantothenic acid content of chicken eggs during embryonic development.

This relatively close relationship between the amount of pantothenic acid in the eggs and the amount in the chicks occurs irrespective of whether the eggs are low or high in pantothenic acid. The fact that pantothenic acid, as shown by the present studies, does not decrease during incubation poses an interesting question as to the function of this vitamin in embryonic development. Gillis, Heuser, and Norris (1943) have shown that the hatchability of eggs from hens on a low level of pantothenic acid may decline to 10%. The necessity of pantothenic acid for embryonic development, and the fact that it does not decrease during incubation, suggests that it may enter into an enzyme system essential for embryonic development.

The differences in the pantothenic acid content of the eggs in Groups I and II, and Groups II and III were highly significant. Likewise, the differences in the pantothenic acid content of the chicks in Groups I and II, and II and III were highly significant. The hens in Group III receiving a ration containing 15.7  $\mu\text{g}$ . of pantothenic acid per g. produced eggs containing an average of 14.0  $\mu\text{g}$ . per g. On the low level of pantothenic acid intake (3.9  $\mu\text{g}$ . per g.) the average amount of pantothenic acid in the eggs was 4.9  $\mu\text{g}$ . per g. From these data, it appears that when liberal amounts of pantothenic acid are fed, there is a tendency to transfer slightly less to the egg than is provided in the feed. When the intake of pantothenic acid is inadequate for optimum reproduction, there appears to be a tendency to transfer a higher percentage of the ingested pantothenic acid to the egg.

Since chicks from eggs having a low initial pantothenic acid content have small reserves of this vitamin, it is possible that the dietary requirements of such chicks for pantothenic acid during the first few weeks after hatching may be higher than for chicks from eggs produced by hens receiving an adequate amount of pantothenic acid in their diet.

The authors are indebted to Professor C. B. Godbey for statistical treatment of the data in this paper.

### SUMMARY

During embryonic development of the chick, the amount of pantothenic acid tends to maintain a constant level, irrespective of the amount in the egg at the beginning of incubation. Hens fed diets containing 3.9, 8.6, and 15.7  $\mu\text{g}$ . of pantothenic acid per g. produced eggs containing an average of 4.9, 7.9, and 14.0  $\mu\text{g}$ . of pantothenic acid per g. The average pantothenic acid content of newly hatched chicks for the corresponding groups was 5.2, 8.3, and 13.6  $\mu\text{g}$  per g.

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# Precipitation and Recovery of Mold Protease by Certain Forms of Lignin and Tannin

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Received May 10, 1945

Lignin sulfonic acid solutions have a marked affinity for certain proteins and may be employed for their precipitation (1, 2). The alkali cook lignin also forms insoluble complexes with such proteins as casein, gelatin, or various cereal proteins (3). The protein-lignin complex is soluble in alkali, and becomes increasingly insoluble as the solution is acidified. The reaction is reversible, and the complex is re-solubilized as the pH is again shifted toward the alkaline range.

In the current study, the affinity of lignin to protein and the reversible solubility and insolubility of the lignin-protein complex was applied to the precipitation and recovery of enzymes from solution. The lignin was a concentrated material derived from the alkaline cook process (4) which forms insoluble protein complexes at mild acidities (pH 4.0–5.0) on the addition of mineral acids. These complexes become soluble as the pH is again shifted in the direction of neutrality.

In the case of lignin sulfonic acid a higher degree of acidity was required for precipitation of the protein-lignin complex. This material, accordingly, is less suitable for the precipitation of enzymes than the alkali cook lignin since many enzymes, including those studied in this paper, are readily inactivated by acid.

The enzymes investigated included mold protease, malt diastase, mold amylase, pancreatic protease, and papain. It was found possible under suitable conditions to precipitate virtually all of the enzymatic activity from the solution in the form of a lignin complex and later to re-dissolve the enzyme at a slightly alkaline pH so as to reconstitute its full activity.

As an example of the procedure, a mold protease derived from a culture of *A. flavus* Link grown on bran is studied in respect to the

effect of lignin concentration and pH on its precipitation and recovery. In dried form, the precipitated complex remained stable after several months of storage at room temperature.

In view of the reported structural likeness of the lignins to tannin and their similar tanning properties (5), it was of interest to determine whether reversible enzyme-tannin complexes could be formed in a similar manner, without affecting the power of the enzyme. It was found that tannic acid and certain types of tannin resemble the alkali cook lignin in their behavior.

### MATERIALS AND METHODS

The following lignin preparation and its analysis was supplied by the Meadol Company, Chillicothe, Ohio:

Lignin . . . . .	99%
Ash . . . . .	0.3%
Solubility in boiling distilled water . . . . .	3%
Methoxyl content . . . . .	21.5%
Hydroxyl content . . . . .	8.5%
Melting point . . . . .	210°C.
Elementary analysis:	
Carbon . . . . .	64.4%
Hydrogen . . . . .	5.9%
Oxygen . . . . .	29.7%

The enzyme preparation was an aqueous extract of *Aspergillus flavus* after a seven-day growth on bran at 20°C. which corresponded to the maximum enzyme production of the mold.

The enzyme extract was prepared as follows: 85 g. of bran were mixed with 80 cc. of H<sub>2</sub>O and placed in a 2½ liter Fernbach flask. The flask was sterilized for 40 minutes at 15 pounds pressure, cooled, and inoculated with a mold that had been grown on a slice of string bean. At the end of the growing period, the mold culture was extracted on a shaking machine with 1250 cc. of tap water plus 20 cc. of toluol. This treatment was sufficient to extract virtually all the enzyme present in the mycelium.

At the end of the extraction the bran was strained off, and the extract filtered through a pad of filter-cel to yield a clear solution. The celite filtration did not affect the enzyme concentration of the solution. The extract so formed remained stable in tryptic power at room temperature for at least six hours.

Since of necessity a number of different enzyme extracts from the mold were employed in the course of these experiments, enzyme precipitations and recoveries are expressed in terms of percentage activity of their respective original solutions prior to lignin treatment. The enzyme extracts, however, showed no substantial differences from batch to batch.

The tannin preparation included samples of mimosa, sumac, and quebracho crystals received from the American Dyewood Company, Chester, Pennsylvania,

and tannic acid derived from gall nuts. A liquid concentration of commercial wattle tannin was received from the Tannin Products Export Corporation, New York City. Two samples of spruce tannin, a liquid concentrate and a powder, were received from the Robeson Process Company, Erie, Pennsylvania.

Enzyme activity was tested for tryptic activity by the method of M. L. Anson (6), which involves determining the depth of color formed with the tryptic-split products of hemoglobin with an alkaline phenol reagent. For the determination we employed a Klett-Summerson photoelectric colorimeter with a red No. 66 filter. The strength of the enzyme solution was determined against the control in which the hemoglobin substrate had been precipitated with trichloroacetic acid before the enzyme addition, the control being adjusted to a zero reading. Difco hemoglobin was used as the substrate and found to give close checks. The Anson method (6) was also applied to pancreatic protease and papain. The Lintner tube series method was employed for  $\beta$ -amylase (7).

Activity of the tryptic mold enzyme was tested in duplicate on a 1:10 dilution of the original extract, generally corresponding to a photometric reading of 350 to 400. Readings on the precipitated enzyme were made after reconstitution to the original volume in the same manner.

Different quantities of 5% lignin dissolved in N/10 NaOH to a pH of approximately 10.5 were added to 50 cc. of the enzyme extract. Adjustment of pH was made by addition of 5% H<sub>2</sub>SO<sub>4</sub>. Both the precipitant and the acid were added dropwise with vigorous stirring. The total volume was adjusted to 55 cc. with distilled water and centrifuged at 3000 r.p.m. for 15 minutes after which the enzyme-lignin precipitate was solidly settled. The activity remaining in the supernatant solution was determined in a 1:10 dilution. Tannin precipitations were similarly carried out. The tannic acid was dissolved in N/10 NaOH to a pH of 8.0; the tannin crystals were readily soluble without alkali addition and were dissolved in distilled water.

In order to avoid inactivation resulting from increase of the acidity on removal of water during drying, the wet precipitate was mixed with small quantities of sodium bicarbonate sufficient to neutralize the acidity in the enzyme-lignin complex. The mixture was dried on an enamel tray or glass plate in a current of air. Slow drying, resulting from thick or watery layers, caused some enzyme inactivation. Drying may be speeded by admixture of a carrier substance, for example, sodium sulfate or a lactose.

The addition of such substances is not necessary, however, when the precipitate is thinly spread, and has been avoided in these experiments.

## EXPERIMENTAL

To determine the optimum pH range for the recovery of the mold protease, a series of precipitations were carried out at pH values from 6.0 to 3.7. To 50 cc. portions of the mold extract were added 2 cc. of 5% alkali cook lignin. The pH was adjusted by the dropwise addition of 5% sulfuric acid to the agitated solution. Determination of the tryptic power of the dried centrifuged precipitate and of the supernatant solution was carried out by the Anson method at pH 7.5.

The activity of the respective dried precipitates and of the supernatant solution is illustrated in Fig. 1. It will be seen that virtually complete precipitation occurs in the pH range 4.0–4.2, at which range full reconstitution is possible. At a lower range, rapid inactivation occurs preventing full reconstitution. At pH 3.7, inactivation is virtually complete.

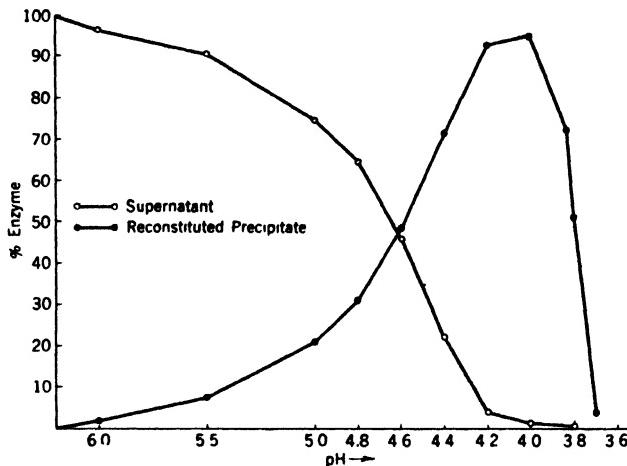


FIG. 1

Influence of pH on the Precipitation of Mold Protease by Alkali Cook Lignin

Variations in the amount of enzyme precipitation according to the concentration of lignin added were studied at pH 4.2 at which optimum recovery had been previously noted. Fifty cc. portions of the mold extract were again employed. An adjustment was made to a pH of 4.2 prior to centrifugation. The dried, neutralized precipitates were tested by the Anson method at pH 7.5.

The results of the quantity variation are illustrated in Fig. 2. It will be seen that the extent of enzyme precipitation is a function of the amount of lignin added, and reaches a maximum at which full recovery is achieved.

To determine whether, and to what extent, variation in lignin concentration would affect the pH range of optimum recovery, comparative precipitations at different lignin concentrations were carried out over a pH range of 6.0–3.5.

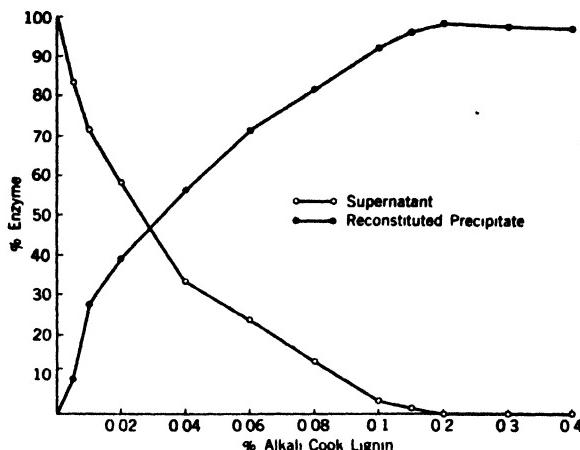


FIG. 2

Precipitation of Mold Protease by Varying Concentrations of Alkali Cook Lignin at pH 4.2

The interdependence of pH adjustment and lignin quantity is illustrated in Fig. 3. Here, activity of the dried neutralized enzyme precipitates is expressed as a percentage of the total activity of the original enzyme extract.

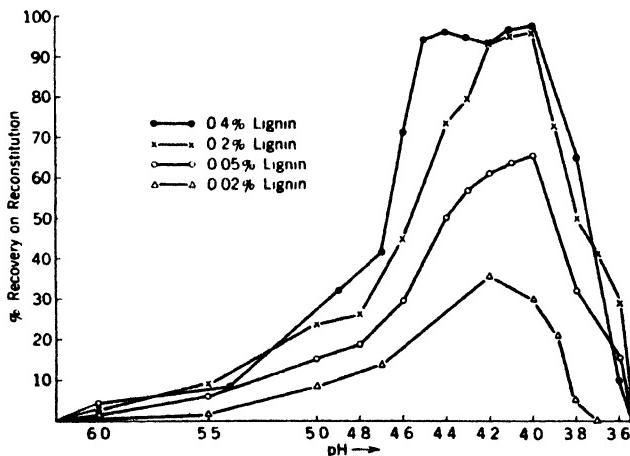


FIG. 3

Comparative Precipitation of Mold Protease by Alkali Cook Lignin at Varying Concentrations and pH Values

Where the highest concentrations of lignin are employed, complete reprecipitation occurs over a relatively wide range (pH 4.5-3.9). At a lower concentration, the range for maximum precipitation is more critical (pH 4.2-3.9). Lesser concentrations fail to achieve complete precipitation, but show their maximum pH action within the same range.

To compare the action of tannic acid to that of lignin, a series of precipitations with 5% tannic acid in *N*/10 NaOH were carried out at pH values ranging from 5.6 to 3.7, 1 cc. of the tannic acid being added to 50 cc. of the enzyme extract. Activity tests were carried out on the dried, neutralized precipitates and on the respective supernatant solutions, as illustrated in Fig. 4. It will be seen that

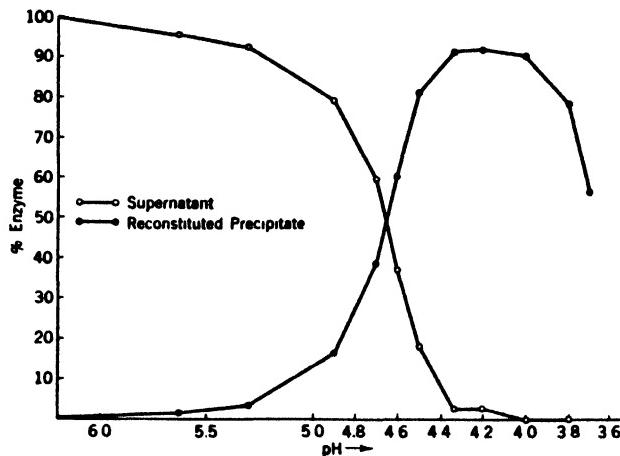


FIG. 4

#### Influence of pH on the Precipitation of Mold Protease by Tannic Acid

the relationship of the precipitability of the enzyme with tannic acid at the several pH values employed is in general similar to that previously determined for alkali cook lignin.

To determine in what manner variations of the tannic acid concentration would influence recovery at various pH values, three concentrations of tannic acid were investigated at pH values ranging from 5.5 to 3.7. In Fig. 5, the tryptic activity of the respective dried neutralized precipitates of tannic acid and mold protease under the various conditions of concentration and acidity are compared.

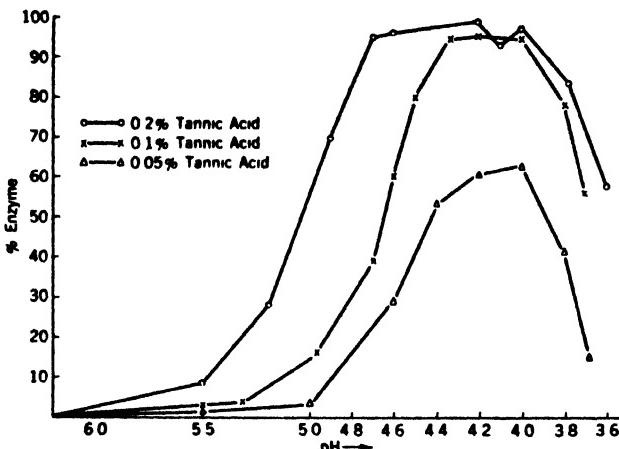


FIG. 5

Comparative Precipitation of Mold Protease by Tannic Acid at Varying Concentrations and Values

The curve shows that in the case of tannic acid, as in the case of alkali cook lignin, there is an interdependence of quantity and optimum acidity, and that in higher concentrations full precipitation can be achieved over a relatively wide pH range (pH 4.0–4.7).

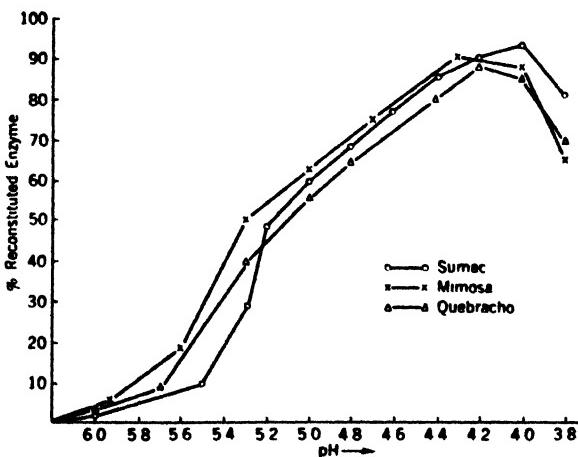


FIG. 6

Comparative Recovery of Mold Protease by Sumac, Mimosa, and Quebracho Tannin

Aqueous extracts of three types of tannin—quebracho, mimosa, and sumac—were studied in regard to their protease precipitating power at a pH range from 6.0 to 3.8. Three cc. of 5% solution of the tannin extract were added in each case to 50 cc. of the mold extract.

Fig. 6 shows the tryptic activity of the dried, neutralized precipitates, the pH values being those at which centrifugation was carried out. It will be seen that the general outline of the curve resembles that previously obtained for tannic acid and alkali cook lignin.

The power to precipitate enzymes without impairing their activity, however, is not apparently shared by all forms of tannin. Negative findings were noted in the case of two commercial preparations of spruce tannin which caused inactivation. Wattle tannin, on the other hand, was found suitable for the protease recovery.

### DISCUSSION

The observation of a reversibly soluble and insoluble complex of mold protease with certain forms of lignin and tannin may be of interest since it is formed without affecting the catalytic properties of the enzyme.

From preliminary observation it appears that the recovery of the enzyme by lignin or tannin can be successfully applied to a number of other proteases and amylases. Successful recoveries were achieved with pancreatic protease, malt diastase, mold amylase, and papain. In each case, the precipitated material was neutralized prior to drying with sodium bicarbonate. Since the malt diastase, however, had been originally tested at pH 5.5, which corresponds to its point of optimum activity, the dried precipitate was dissolved in a phosphate buffer at this pH prior to testing.

It would appear, thus, that lignin- or tannin-precipitated enzymes can be reconstituted to a slightly acid pH without impairing their activity. Such adjustment, however, cannot be carried to a pH much below 5.0 without causing the enzyme-lignin or enzyme-tannin complex to reprecipitate.

Preliminary tests indicate that the enzyme complex can be further purified by extraction of the wet precipitate with certain organic solvents, *e.g.*, ethanol, acetone, or methanol. It appears that only water-miscible solvents are suitable for this purpose. Presumably, the extraction removes portions of the tannin or lignin in the precipitate not in combination with the enzyme, including much coloring matter.

The authors wish to express their appreciation to Dr. Arthur L. Schade for his interest and suggestions; and to Dr. Kenneth Raper for identification of the mold.

### SUMMARY

(1) Aqueous extracts of mold protease and other enzymes may be precipitated from solutions and recovered in dried form by the addition of certain forms of lignin and tannin, followed by partial acidification and the removal of the resulting enzyme complex by centrifugation or filtration.

(2) The pH range of precipitation was found to be of critical influence for lesser concentrations of the precipitant. Where larger amounts of the precipitant were employed, however, full recovery could be achieved over a wider range of acidity.

(3) Drying may be conveniently carried out at room temperature in a current of air, after neutralization of any acidity in the enzyme precipitate by the admixture of an alkaline salt. The dried precipitate is then fully soluble, and may be utilized for enzymatic digestions.

(4) Quantitative relationships of the enzyme recovery in respect to concentration of the precipitant and acidity of the precipitation are illustrated in the case of alkali cook lignin, tannic acid, and sumac, quebracho, and mimosa tannin.

(5) Under appropriate conditions, virtually all of the enzymatic activity may be recovered by this procedure. The dried material was found to be stable over a period of several months.

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## On the Mechanism of Enzyme Action. Part 26

### Evidence of Chemical Blocking of Carboxylase in Fusaria in Nitrate- and *p*-Aminobenzoic Acid-containing Media During Alcoholic Fermentation

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Received May 22, 1945

#### INTRODUCTION

The presence of certain elements or groups in the hypothetical structure of an enzyme may be ascertained either directly or indirectly. Indirect proof of the presence of a group, or its functioning, might be based on observation of the effect of certain reagents on the activity of the enzyme. Accordingly, only such atomic groups can be detected as are essential for the operation of the enzyme. For example, inhibition of enzyme action by HCN is often considered indirect evidence of the presence of iron because of possible formation of an iron complex, determinable by a complete reversal of the enzyme's spectrum. Similar proof is afforded by the spectrum of the H<sub>2</sub>S-complex of catalase although its dissociation is much more rapid than that of the HCN-complex. However, the possibility should be borne in mind that every change made in the enzyme molecule may influence its activity, whether close to or at some distance from the groups responsible for actual reaction with the substrate.

In some recent investigations carried out in this laboratory (1) evidence was educed that although its catalase (2) content is high, both HCN as well as elementary sulfur can be conveniently introduced into fusarial culture media without measurably inhibiting the enzymatic activity of the mold. In fact, the first agent served as an excellent carbon source and the second as an effective hydrogen acceptor. On

<sup>1</sup> Communication No. 42.

the other hand, it was later found that, in cultivating *Fusaria*, nitrates or, to a lesser extent, ammonium sulfate may serve a triple purpose (3). They may be used simultaneously as (a) an indispensable nitrogen source, (b) a substrate itself, and finally (c) as an expedient for clarifying the metabolism of the organism. When fermenting carbohydrates in the presence of nitrates, it was observed that the latter are reduced to hydroxylamine *via* nitrite, while also making the pyruvic acid phase manifest in the course of the degradation of hexoses as well as of pentoses.

Hence, if an attempt be made to correlate the above observations, the following facts should be kept in mind: (a) Judging from preliminary experiments of Loew (4) nitrous acid may be considered as a specific reagent for NH<sub>2</sub> groups present in enzymes. (b) Windaus (5) showed the production of two compounds from thiamine by oxidation with nitric acid. (c) The function of the pyrophosphoric ester of thiamine or co-carboxylase present in or added to growing *Fusaria* possessing a significant NH<sub>2</sub> group in its molecule, and capable of rapidly and continuously destroying pyruvic acid, is fully counteracted (1a, 6) in nitrate-nitrite- and carbohydrate-containing media.

There can be no doubt that nitrous acid will react with these amino groups as well as others, either by exchanging the NH<sub>2</sub> group for hydroxyl, or by giving rise to the formation of a diazo compound (7). Thus, if the proper coupling agent is present and if the concentration<sup>1</sup> of the NH<sub>2</sub> groups available is high enough, it is to be expected that the reaction can be made visible and measurable despite the fact that the diazotization proceeds rapidly.

In recent experiments (8) establishing conditions which control the balance of pyruvic acid accumulation in the presence of nitrite, sulfate, hydroxylamine, etc., in *Fusarium* fermentation, the above mentioned requirement could not be satisfied owing to the low concentration of co-carboxylase in the mold. This difficulty could, however, be circumvented when known amounts of *p*-aminobenzoic acid (PAB) were introduced into media prepared for these fermentations.

This complement, which contains 11.77% NH<sub>2</sub> as contrasted with 5.35% present in the enzyme, was helpful in demonstrating the reaction taking place. Thereby indirect but tangible evidence was furnished that the accumulation of pyruvic acid in these fermentations is caused

<sup>1</sup> One kilogram of fresh yeast contains about 90 mg. of co-carboxylase. The thiamine content of dry FIB amounts to about 20γ/g. (1b).

by the inhibition of the co-carboxylase brought about by chemical interaction between the nitrite obtained from the nitrate present in the medium with the NH<sub>2</sub> group of the co-carboxylase.

In addition to the above experiments the influence of desthiobiotin and of sodium azide on Fusaria growing on glucose was also studied.

### EXPERIMENTAL

*Analytical Methods.* Determinations of pyruvic acid (PA), nitrite, nitrate, carbohydrates and mycelial weights were carried out according to the procedures described in earlier investigations. In the experiments with PAB, however, the nitrite determinations were made in the absence of sulfanilamide. In all other experiments, i.e., in studying the effect of NaN<sub>3</sub> and desthiobiotin, the diazotizations were carried out by using sulfanilamide as a base. Measurements of the hydrogen ion concentration were made electrometrically, using a glass electrode. The media were adjusted with KOH and phosphoric acid.

*Procedures.* All experiments were conducted in 125 ml. Pyrex Erlenmeyer flasks with 50 ml. of medium and were sterilized at 15 lbs. pressure for 20 minutes. The various amounts of PAB were added to the medium *before* sterilization. Desthiobiotin and NaN<sub>3</sub>, on the other hand, were added *after* sterilization of the media with sterile technique.

The mold employed in these experiments was *Fusarium lini* B. (*FlB*) originally obtained from the North Dakota Agric. Experiment Station, Fargo, N. D., and was maintained in agar cultures with a medium referred to in previous investigations. All flasks were inoculated with 1 ml. of a mycelial spore suspension as indicated in earlier studies, and the values obtained represent an average of three flasks.

TABLE I  
*Effect of Various Amounts of PAB on Glucose Fermentations*

Day	B		200,000 $\gamma$		2000 $\gamma$		1000 $\gamma$		200 $\gamma$	
	PA	Mat Wgt.	PA	Mat Wgt.	PA	Mat Wgt.	PA	Mat Wgt.	PA	Mat Wgt.
2	19	—	11	—	18	—	18	—	18	—
3	78	34	31	13	66	34	69	32	78	32
4	111	58	57	45	93	52	105	65	99	40
5	106	63	82	68	99	49	112	71	123	45
7	140	95	95	81	125	83	134	78	140	91

Medium: 20 g. Glucose, 5 g. KNO<sub>3</sub>, 5 g. KH<sub>2</sub>PO<sub>4</sub>, 0.750 g. MgSO<sub>4</sub>·7H<sub>2</sub>O, water to 1 liter.

pH = 4.2.

Recorded amounts are expressed in mg./100 ml.

B = blank.

## RESULTS AND DISCUSSION

*I. Effect of Various Amounts of PAB on Glucose Fermentations in the Presence of  $KNO_3$* 

A preliminary experiment shows that PAB, in quantities ranging from 200 to 2000  $\gamma$  per liter, brought about no increase in the PA accumulation, nor did it influence the growth expressed by mycelial weights. This is clearly indicated in the first two days' analyses in which the PA values are practically identical. Addition of 200,000  $\gamma$ /liter, however, did inhibit fermentation. In good agreement with observations on *Ps. mors-prunorum* (9) and certain molds (10) less

TABLE IIa

*Effect of 200,000 $\gamma$ /liter PAB at Different pH on Glucose Fermentation  
Initial pH*

Day	4 2		5 5		6 5		4 2		5 5		6 5	
	PA accumulated						NO <sub>2</sub> accumulated					
	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB
2	9.0	0.0	8.0	3.0	6.3	3.8	34.0	0.0	0.0	0.0	2.0	2.3
3	51.0	6.0	33.0	18.8	22.2	16.5	17.2	108.2*	35.2	42.0	14.0	1.8
4	61.0	15.0	61.0	45.0	42.0	33.8	5.2	115.0	9.0	58.4	6.0	2.3
5	76.0	18.8	72.0	54.6	64.5	43.5	7.0	43.0	1.0	4.8	7.6	2.3
6	92.0	21.0	90.0	66.0	87.0	80.2	6.2	44.0	1.0	4.0	3.0	2.0
8	98.0	30.0	87.0	72.0	115.5	69.7	3.0	43.2	4.2	14.0	1.0	2.0

Day	4 2		5 5		6 5		4 2		5 5		6 5	
	Mat weight						Glucose fermented					
	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB
2												
3	29.4	19.0	27.0	28.1	29.0	38.0						
4	71.4	33.8	51.9	47.5	27.6	37.2						
5	101.9	49.0	70.4	75.7	45.5	54.0						
6	94.5	59.7	60.0	62.5	84.0	74.7	0.56	0.55	0.57	0.45	0.33	0.40
8	115.5	73.8	98.8	92.7	72.0	84.0	0.71	0.79	0.80	0.68	0.51	0.54

\* Diazonium compound present.

TABLE IIb  
*Trend of pH Changes and Nitrate Available*

Day	pH						Nitrate					
	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB
0	4.2	4.0	5.5	5.3	6.7	6.6	8.0	8.1	8.0	8.1	8.0	8.1
5	4.4	5.2	4.8	4.8	6.3	6.3						
8	5.4	5.6	5.1	5.1	6.2	6.1	6.6	7.7	7.2	7.2	7.6	7.8

Medium: 40.0 g. Glucose, 5.0 g.  $\text{KH}_2\text{PO}_4$ , 0.75 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 8.00 g.  $\text{KNO}_3$ , water to 1 liter.

B = blank.

PA and mycelium were found, and a transient accumulation of the diazonium compound of PAB was qualitatively detected on the third day of growth by adding 1 ml. of 0.1% solution of the coupling agent, *N*-(1-naphthyl)-ethylenediamine dihydrochloride in water, to 5 ml. of medium (Table I).

#### *II. Effect of 200,000 γ/liter PAB on Glucose Fermentation in the Presence of $\text{KNO}_3$ at Varying pH Values*

In the next experiments the effect of 200,000  $\gamma$  per liter of PAB was investigated to study the possible influence of the diazonium compound formed during fermentation at different hydrogen ion concentrations. The extent of nitrite accumulation was simultaneously determined. From the data recorded in Table II it can readily be seen that the PA accumulation in the control varies with hydrogen ion concentration, pH 4.2 giving rise to more PA than pH 5.5 and 6.7. On the other hand, the effect of PAB on the accumulation of the keto acid is reversed: less PA and less growth are observed at pH 4.2 than at pH 5.5 and 6.7. The diazonium compound was detected in those experiments in which the  $\text{NO}_2^-$  accumulation was higher.

This observation stimulated the thought that this compound, the formation and stability of which is strongly dependent on the acidity of the medium, could exert an influence on the growth of the organism, since PAB itself is regarded (11) as an essential metabolite associated with certain enzymatic processes in bacterial growth. A series of quantitative determinations of the diazonium compound actually

formed were carried out, therefore, at different pH values ranging from 3.4 to 6.5 in the presence of PAB amounts varying from 2000  $\gamma$  to 1.0 g./l.

TABLE III  
*Relation between Nitrite Formed and Diazonium Compound Detected*  
Initial pH

Day	3 4				4 4			
	blank	2 PAB	200 PAB	1000 PAB	blank	2 PAB	200 PAB	1000 PAB
2	30.0	15.6	none	none	52.0	55.0	1.0	1.4
3	26.0	20.4	39.6 <sup>1</sup>	1.8	35.6	15.8	101.0 <sup>2</sup>	4.4
4	4.4	3.6	54.0 <sup>3</sup>	3.6	3.4	4.4	90.0	8.4
5	—	2.2	39.6 <sup>4</sup>	18.6 <sup>5</sup>	2.4	2.0	44.0	40.0
7	3.7	2.0	34.0 <sup>7</sup>	31.6 <sup>8</sup>	5.6	3.7	36.4	96.4

Day	5 5				6 5			
	blank	2 PAB	200 PAB	1000 PAB	blank	2 PAB	200 PAB	1000 PAB
2	2.4	3.0	2.0	none	3.0	10.0	3.6	1.8
3	2.4	3.0	2.0	3.0	2.0	2.0	2.0	2.4
4	9.2	10.0	20.6	4.4	2.3	2.0	2.0	2.3
5	2.4	3.7	12.4	26.0 <sup>6</sup>	1.0	none	1.0	none
7	3.5	3.6	9.2	47.6 <sup>9</sup>	62.3	62.0	118.4 <sup>10</sup>	74.0

Diazonium compound present:

<sup>1</sup> 8.4 $\gamma$ , <sup>2</sup> 22.0 $\gamma$ , <sup>3</sup> 4.4 $\gamma$ , <sup>4</sup> 2 $\gamma$ , <sup>5</sup> 2.4 $\gamma$ , <sup>6</sup> 3.5 $\gamma$ , <sup>7</sup> 1.0 $\gamma$ , <sup>8</sup> 3.5 $\gamma$ , <sup>9</sup> 3.5 $\gamma$ , <sup>10</sup> 4.4 $\gamma$ .

Results expressed in  $\gamma$ /100 ml. medium as in previous experiments.

PAB amounts expressed in mg.

From the data recorded it can be seen that the amount of diazonium compound detected is closely related to the pH of the medium, and the quantity of PAB added to it. It can also be recognized that the inhibition caused by higher amounts of PAB is accompanied by an increased accumulation of  $\text{NO}_2^-$  which in turn indicates that the HCN-insensitive reductase and dehydrogenases present in *Fusaria* are affected, whereby the reduction of  $\text{NO}_2^-$  to hydroxylamine is delayed.

Depending on the concentration of the PAB the formation of a yellow pigment was also observed in these experiments in the second

week, indicating the presence of a powerful PAB-oxidase in the perfectly white mycelium of FlB.

*III. Effect of 200,000  $\gamma$ /liter PAB in the Presence of Varying Nitrogen Sources on Glucose Fermentation*

To study the effect of PAB on glucose fermentation, experiments were carried out in the presence of  $\text{KNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ , and  $\text{NH}_4\text{NO}_3$ , assuming that an interaction between some of these nitrogen sources and the PAB would throw further light on a possible growth inhibition caused by the diazonium compound formed. The nitrogen sources were employed with equivalent nitrogen values and the same spore suspensions were used for inoculation.

TABLE IV

*Effect of 200 mg./liter of PAB on Glucose Fermentation in the Presence of  $\text{KNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$ , and  $\text{NH}_4\text{NO}_3$  at pH 4.2*

Day	PA accumulated						Mat weight					
	$\text{KNO}_3$		$(\text{NH}_4)_2\text{SO}_4$		$\text{NH}_4\text{NO}_3$		$\text{KNO}_3$		$(\text{NH}_4)_2\text{SO}_4$		$\text{NH}_4\text{NO}_3$	
	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB
3	72	30	24	13.0	64	19	50.7	20.0	42.1	22.0	41.0	16.8
6	129	75	22	6.0	131	45	142.5	126.1	84.5	85.3	92.4	94.9

Day	Glucose fermented						Final pH					
	$\text{KNO}_3$		$(\text{NH}_4)_2\text{SO}_4$		$\text{NH}_4\text{NO}_3$		$\text{KNO}_3$		$(\text{NH}_4)_2\text{SO}_4$		$\text{NH}_4\text{NO}_3$	
	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB	B	PAB
3	—	—	—	—	—	—	—	—	—	—	—	—
6	1.70	0.46	1.15	1.06	1.28	1.05	4.0	4.5	2.9	3.0	4.3	4.4

Results expressed in mg. per 100 ml. of medium.

Medium: 40.0 g. glucose, 5.0 g.  $\text{KH}_2\text{PO}_4$ , 0.75 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 8 g.  $\text{KNO}_3$  or 5.22 g.  $(\text{NH}_4)_2\text{SO}_4$  or 6.34 g.  $\text{NH}_4\text{NO}_3$ , water to 1 liter.

B = blank.

Examining the data recorded in Table IV, it can be concluded that practically the same inhibition caused by PAB is also noticeable in the experiments with ammonium sulfate-containing media as well as with the other nitrogen sources. It is therefore, to be assumed that an inhibition, if any, brought about by the diazonium compound formed or accumulated because of reaction of the  $\text{NO}_2^-$  formed with the amino group present in PAB, should be considered negligible. This observation appears to be more significant inasmuch as it also shows that an inhibitory action can be evidenced without any apparent alteration of the structure of PAB.

*IV. Effect of Increasing Amounts of PAB on Xylose Fermentations with  $\text{KNO}_3$  as a Nitrogen Source*

The inhibition brought about by 200,000  $\gamma$  per liter of PAB and the possible action of smaller quantities was also studied in xylose

TABLE V  
*Effects of Various Amounts of PAB on Xylose Fermentation at pH 5.2  
in the Presence of  $\text{KNO}_3$*

Day	B	PA accumulated in mg /100 ml				
		50 $\gamma$	500 $\gamma$	5000 $\gamma$	30,000 $\gamma$	200,000 $\gamma$
3	13	16	13	15	10	7
4	20	20	18	18	14	13
5	19	21	25	24	15	14
6	21	25	22	25	17	18
7	28	30	32	32	24	33
Day	B	NO <sub>2</sub> accumulated in $\gamma$ /100 ml				
		50 $\gamma$	500 $\gamma$	5000 $\gamma$	30,000 $\gamma$	200,000 $\gamma$
3	16.4	13.6	11.4	16.4	28.6	32
4	7.0	7.6	9.2	10	13.8	19.6
5	8.2	11	11	14	15.6	17.6
6	4.4	2.6	3.6	2	4	14.0
7	10	9.2	9.4	10	12	17.8
Day	B	Mycelium weight in mg				
		50 $\gamma$	500 $\gamma$	5000 $\gamma$	30,000 $\gamma$	200,000 $\gamma$
3	38	39	46	42	38	15
4	57	59	62	61	64	45
5	74	71	75	73	72	69
6	86	76	75	84	91	91
7	90	86	86	90	99	105

Medium: 20.0 g xylose, 5.0 g  $\text{KH}_2\text{PO}_4$ , 0.75 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 8 g  $\text{KNO}_3$ , water to 1 liter.

Final pH: ~ 5.2

B = blank.

fermentation. In these experiments the inhibition of the higher quantity of PAB was somewhat less marked than in the glucose experiments. No diazonium compound had been detected on the third day of analysis and the initial inhibition disappeared during the following days.

#### *V. Inhibitory Action of NaN<sub>3</sub> in Fusarium Fermentation*

The action of varying amounts of sodium azide were tried in Fusarium fermentation. The addition of 100,000 γ and 10,000 γ per liter upon inoculation proved to be completely inhibitory to growth while amounts of 500 γ per liter caused partial inhibition. The higher amounts, when administered on the third and sixth day also brought about partial inhibition. This indicates too that the respiratory system in the mold is strongly affected.

#### *VI. Desthiobiotin in Fusarium Fermentation*

The action of varying and increasing amounts of desthiobiotin on Fusaria did not show any influences on the growth of the mold. However, higher quantities seem to have a slight inhibitory action. This observation is in agreement with earlier investigations (6) the results of which indicated that biotin is not a growth factor for Fusaria.

#### ACKNOWLEDGMENTS

This study was carried out with the aid of a grant from the Rockefeller Foundation. Desthiobiotin was obtained through the courtesy of Dr. H. M. Wuest, Hoffmann-LaRoche, Inc., Nutley, N. J.

#### SUMMARY

1. The action of PAB on growing Fusaria varies with the pH of the media.
2. Independent of the nitrogen source but depending on the quantity of PAB present in fusarial glucose and pentose fermentations, the rate of PA accumulation is unaffected by low amounts or inhibited by amounts above 30,000 γ. These amounts of PAB are also inhibitory for dehydrogenases and reductase present in Fusaria.
3. In Fusaria fermentations of glucose in the presence of KNO<sub>3</sub> and 200,000 γ PAB or more, an interaction can be rendered noticeable between the transient NO<sub>2</sub> group and the NH<sub>2</sub> group present in the base by the coupling of the diazonium compound formed.

4. The diazotization of PAB by  $\text{NO}_2^-$  formed in *Fusarium* fermentation may be regarded as indirect evidence that the abundant accumulation of PA in the course of hexose and pentose degradations is due to an inhibition of the carboxylase because of reaction of its  $\text{NH}_2$  group with the  $\text{NO}_2^-$  of the media.

5. If the culture media contain  $(\text{NH}_4)_2\text{SO}_4$  as the nitrogen source no coupling can be demonstrated. It is, therefore, improbable that, in addition to the evidenced inhibitory effect of PAB, an additional inhibition would arise which may have been caused by the transient diazonium compound formed.

6. As with biotin, varying amounts of desthiobiotin have no significant effect on the rate of *Fusarium* fermentations.

7.  $\text{NaN}_3$  is a strong inhibitor in *Fusarium* fermentations.

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# The Heterogeneity of Amylose and Amylopectin, Part I

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Received May 29, 1945

## INTRODUCTION

Starch is composed of glucopyranose polymers which can be fractionated into two components which differ in respect to axial ratio (1-5). The fraction which exhibits certain characteristics of a linear polymer is now generally referred to as amylose and that which is presumably composed of nonlinear polymers as amylopectin. This usage is consistent with, and a logical development of, the proposal of Maquenne and Roux (6) to call that fraction amylose which is composed essentially of maltose residues and that fraction amylopectin, the structure of which is more complicated. Unsettled questions relating to the composition and structure of starch are the proportion of linear and nonlinear polymers in the various starches and an explanation for the difference in the axial ratio between the two fractions. Considerable evidence supports the view that a complexity arises from branching in the structure of the amylopectin molecules and that the branch is formed by a 1-6, $\alpha$ -glucosidic linkage. In pursuing this view other questions have arisen. Are the supposedly linear polymer fractions of the starches composed only of unbranched chains of glucopyranose units? Are the amylopectin fractions composed of molecules containing only one pattern of branching in their structure? (Cf. 1, 7, 8, 9, 10.)

This paper presents additional data to show that the linear polymer fractions of several starches differ from each other (4, 5, 11), to show that the branched polymer fraction of starch is heterogeneous as is also the linear polymer fraction (11, 12, 13), and to show that these differences, and the heterogeneity noted, are inadequately explained by possible variations in molecular magnitude alone.

Differences in the reported estimates of the proportion of amylose and amylopectin fractions in any one starch are largely the result of

differences in definitions and of analytical procedures. One of the more acceptable of these analytical methods is based upon the precipitability of the amylose fraction from a dispersion of starch in water by the addition of higher alcohols (14). The fraction which is precipitated is arbitrarily taken for the purpose of this discussion as amylose and that which is more soluble as amylopectin.

## EXPERIMENTAL

### *Sub-Fractionation of the Amylose of Corn Starch*

Three arbitrarily defined subfractions of the amylose fraction may be prepared by a combination of hot water extraction of whole starch and alcoholic precipitation of the amylose from these extracts.

Commercial corn starch was defatted (15, 16) from an original total fat content of 0.92% to 0.25% by refluxing with 85% methanol (17) and then thoroughly washed with cold water. On a dry weight basis, 500 g. of the starch was extracted with 20 l. of water by gentle stirring at 75°C. for one hour and was filtered while hot and 54.57 g. of starch solids were recovered in the filtrate (Table I).

The insoluble residue was made up with water to the original dilution for a second extraction which was made at 90°C. by gentle stirring for one hour. The mixture was filtered while hot and 23.02 g. were recovered in the filtrate. Both extractions were made at pH 6.2-6.4 and filtrations were by gravity and under toluene.

The residue remaining insoluble after the extraction at 90°C. was solubilized in 6 liters of 0.67*N* NaOH by stirring at 25°C. for 6 hours, then diluted to about 19.5 l. and allowed to digest overnight. The solution was acidified to pH 6.0 with HCl and adjusted to a volume of 20 liters. The solution was heated to 80°C., saturated with butanol and allowed to cool slowly to room temperature over a period of 72 hours. The precipitated solids were recovered by use of a supercentrifuge at 40,000 r.p.m. All the amylopectin fractions remaining were discarded.

The two hot-water extracts were separately concentrated by vacuum distillation to solutions which contained approximately 1% solids, were warmed to 80°C., filtered hot, and saturated with butanol. The solutions were allowed to cool slowly to room temperature over a period of 48 hours and then centrifuged at 2000 r.p.m. to collect the precipitates.

The three amylose precipitates were separately purified by two recrystallizations. They were washed with cold water, saturated with butanol and then dissolved in hot water to make approximately 1% solutions, and filtered at 80°-90°C. The filtrates were saturated with butanol. After slowly cooling at room temperature and collecting the crystals by centrifuging, the recrystallization procedure was repeated. All three lots of crystals were washed repeatedly with ice water saturated with butanol and were then dehydrated with four successive portions of 1200 cc. of absolute methanol in closed vessels over a 7 day period. The dehydrated crystals were freed of methanol in a desiccator by evacuation for a prolonged period of time over  $H_2SO_4$  in a vacuum desiccator. The fractionation data on yield are summarized in Table I.

TABLE I  
*Fractionation of Amylose from 500 g. of Partially Defatted Corn Starch*

	Water extraction at 75°C.	Grams of solids	Water extraction at 90°C.	Water-insoluble at 90°C.
Extracted	54.570	23.020		
In first mother liquor	5.611	4.058		
In first wash	0.585	0.417		18.260
In second mother liquor	3.369	0.236		5.762
In third mother liquor	2.492	1.051		2.925
In final wash of crystals	0.012	0.066		0.063
Yield of crystals	42.2	14.1		23.7

*Sub-Fractionation of the Amylopectin Fraction of Corn Starch*

A butanol-non-precipitable fraction from defatted corn starch (18) was dissolved in hot water by slowly stirring 30 g. of the dry product into 1000 cc. of warm water and then raising the temperature under reflux to about 80°C. When solution was complete, 200 cc. of butanol were added and then slowly with stirring, 180 cc. of methanol. The solution was cooled to room temperature over a period of 72 hours. The fine grained precipitate was readily centrifuged, washed, and recrystallized twice from a solution which contained the same proportion of water, butanol, and methanol as was used in the first precipitation. Recrystallization was performed by dissolving the precipitate in 700 cc. of water at 80°C., adding 140 cc. butanol and then, slowly, 126 cc. methanol. The yield of sub-fraction I was 14 g.; alkali (labile) number, 3.2; linear fraction by iodine titration, 11.8%; by spectrophotometric analysis, 13%;  $\beta$ -amylase conversion limits to maltose after the crystallization and two recrystallizations were respectively 58.7%, 60.9%, and 59.6%.

The washes and mother liquors were combined and evaporated to a small volume by vacuum distillation and diluted to one liter. After heating to 80°C. in a closed vessel provided with a reflux condenser, 242.5 cc. of butanol was added and then

TABLE II  
*Treatment of Amylopectin in Subfraction II with Cotton*

Solution	Dry substance subfraction in solution	Volume of solution treated	Amount of cotton added	"Linear" by iodine titration	Conversion limit to maltose by $\beta$ -amylase
Original	g./100 cc. 1.041	cc. 1600	g. 75	per cent 8.33	per cent 56.1
After 1st cotton adsorption	1.072	950	47.5		
After 2nd cotton adsorption	1.081	675	34		
After 3rd cotton adsorption	1.087	400		7.93	55.9

200 cc. of methanol. A granular precipitate formed which was washed with a combination of 100 cc. water, 24.25 cc. butanol, and 20 cc. methanol. The yield of subfraction II was 8 g.

A sample of this product was treated further in water solution with a large amount of cotton in order to remove any possible amylose contamination (4, 8, 11, 19, 20). To a 1% water solution of the subfraction at pH 5.0, 4.7 g. of cotton (prewashed with methanol and water) were added per 100 cc. of the solution treated. The mixture was stirred for one hour at room temperature, and the cotton was pressed out and removed. The liquor was treated twice more with the same ratio of cotton to solution. The solution was then filtered and the subfraction was precipitated by the addition of butanol and methanol. Data on the purification are given in Table II.

After the removal of the second subfraction, the balance of the amylopectin solids were recovered from solution by the addition of two volumes of methanol. A gummy mass gradually settled out on standing. This was recovered by decantation. The gum was dissolved in a small amount of water, and the clear solution was poured slowly, with vigorous stirring, into a large volume of methanol. A fine, powdery product resulted. This and all other subfractions were repeatedly washed with absolute methanol and dried by procedures previously given. Subfraction III: yield, 6 g.;  $\beta$ -amylase conversion limit, 50 to 52%; alkali (labile) number, 13.7; per cent "linear" by iodine titration, zero.

Treatment with cotton failed to change any characteristic of subfraction III. It stained a wine red color with iodine and it has been used as a standard of reference (as 100% amylopectin) in spectrophotometric analysis (9).

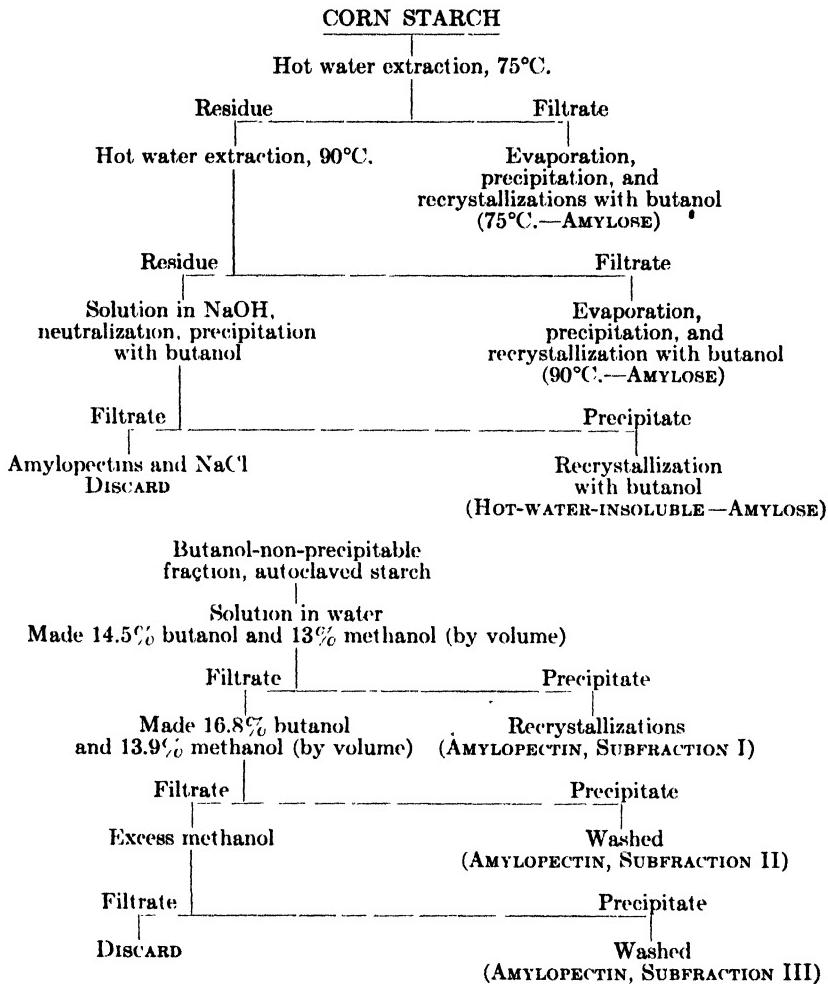
The origin of the six corn starch subfractions described are reviewed diagrammatically (page 381).

#### *Preparation of Crystalline Amyloses*

Tapioca crystalline amylose was prepared by procedures similar to those given for the preparation of corn and potato crystalline amyloses (11). Five hundred grams of "Sando" tapioca starch, thoroughly prewashed with cold water, was extracted with 20 l. of water by gentle stirring for one hour at 60°C. at a pH 6.3. Twenty-nine grams dissolved and was recovered in the filtrate after gravity filtration, of which 21.4 g. precipitated when the solution was concentrated *in vacuo* to a 1% solution, filtered, saturated with butanol and allowed to cool slowly to room temperature. The crystals were recovered by centrifuging, washed with a small amount of ice water, saturated with butanol, and recrystallized twice by dissolving in two liters of hot water, filtering and saturating the filtrate with butanol. In the first recrystallization 1.00 g. remained soluble in the mother liquor and in the second, 0.31 g. Twenty grams of product was dehydrated by five washes of one liter each of absolute methanol and then dried in a vacuum desiccator over sulfuric acid.

#### *Characterization of Starch Fractions*

Alkali (labile) numbers were determined according to the method of Schoch and Jensen (21). The conversion limits to maltose by  $\beta$ -amylase were determined for several fractions by the method of Kerr and coworkers (11, 22). It should be pointed out, however, that this method is unreliable for certain amylose fractions owing to retrogradation effects which may occur during the determination (11).



The determination of the percentage of linear material in the fractions was made by a modification of the iodine titration method of Bates, French, and Rundle (4). A weighed quantity (between 50 and 200 mg.) was dissolved in 2.5 cc. of 2*N* KOH at room temperature. When solution was complete, 7.5 cc. of water was added. The solution was neutralized with 0.5*N* HCl (methyl orange), warmed to 90°C., cooled, and mixed with 5 cc. of *N* KI. The solution was then diluted to 100 cc. and titrated, potentiometrically by means of a Coleman Electrometer, with a 0.001*N* iodine solution which was also 0.05*N* to KI and KCl. It was assumed that the potential is developed by the presence of free iodine. A standardization curve was

prepared (without starch) from which the potentials developed by various amounts of free iodine may be determined. The iodine absorbed by the starch fraction was calculated for each addition of iodine by subtracting the free iodine from the total amount of iodine added. Free iodine was then plotted against absorbed iodine. The amount of absorbed iodine increases rapidly and almost linearly during the first part of the titration and until absorption is very nearly complete. Then the curve breaks abruptly. This point is estimated graphically and the percentage of iodine absorbed was calculated. Since the crystalline amyloses of corn, potato, and tapioca starches absorb 20.0%, 20.2%, and 20.1% of iodine, respectively, the percentage of linear-material in the unknown was estimated by dividing the percentage of iodine absorbed by the unknown by 0.20.

Not only has the presence of traces of extraneous fat been found to give low values by iodometric methods, but low values are also obtained on certain materials when the strength of alkali used by former investigators is employed. This is particularly true of amylose fractions.

The spectrophotometric method for the determination of the linear polymer content of starch and its fractions has been given by Kerr and Trubell (9).

The solution viscosities of the fractions were determined in ethylenediamine (Eastman Kodak Company, 95%). The viscosity of the solvent was determined daily before each series of tests. A modified Ostwald Viscometer (5 cc.) which gave a value for water of 136.5 sec at 25°C. was used. The solvent varied between the limits of 282 and 288 seconds. An accurately weighed sample (corrected for moisture) was introduced into a glass stoppered volumetric flask and covered with solvent. The flask was closed and placed in a desiccator until solution was complete. This usually required 15 to 20 hours. The flask was then filled to the mark (at 20°C.), shaken, and 10 cc. of the solution was introduced into the viscometer. The latter was protected at both ends with tubes filled with soda-lime. The viscosities were determined in a water bath at 25°C.  $\pm$  0.05. Although the viscometer had a relatively low flow rate and readings varied between 282 and 600 seconds, the average deviation from the mean in the several determinations was less than one part per thousand, if over three parts per thousand, the determination was discarded.

## DISCUSSION

A study of the solution viscosity of high polymers has attracted considerable interest since Staudinger, Kraemer, and others pointed out that the data could be used to gain a better understanding of the size and shape of these very large molecules. For example, in three recent reviews, by Fordyce (23), Lauffer (24) and Huggins (25) in which the work of a number of investigators is analyzed and co-ordinated, it is apparent that (a) Intrinsic viscosity,  $[\eta]$ , is a function of the chain length of a homologous polymer series in a given solvent under fixed conditions. (b) In comparing similar polymers under identical conditions,  $[\eta]$  is a function of axial ratio. (c) The values derived from viscosity are in most cases close to the weight-average

values, and therefore, the results for a member of high polymer series are little affected by the presence of substantial quantities of members of the series which are of very much less molecular magnitude. (d) A linear function is obtained when  $\eta_{sp}/C$  is plotted against specific viscosity,  $\eta_{sp}$ , where  $C$  is the concentration of the solute. Huggins (26, 27, 28) has indicated why this function should be linear, and Foster and Hixon (5) have demonstrated the fact for starch fractions in ethylenediamine. Our data have been plotted in this manner (Fig. 1) and  $[\eta]$  taken as the intercept of the curve on the  $\eta_{sp}/C$  axis.

Furthermore, according to Huggins the slope of the curve, plotting  $\eta_{sp}/C$  against  $\eta_{sp}$ , is characteristic for a given solvent-solute system. An equation may be written:

$$k = \frac{\eta_{sp}/C - [\eta]}{[\eta]\eta_{sp}}$$

in which the constant  $k$  serves to characterize the solute-solvent system and this constant is the same for all members of a polymer series irrespective of chain length.

Bartovics and Mark (29) found that this was approximately the case for carefully fractionated cellulose acetates, irrespective of the chain length, when their solutions in acetone were studied. However, since fractions of polystyrene, which were prepared at three different polymerization temperatures and dissolved in the same solvent gave different values for  $k$ , Alfrey, Bartovics, and Mark (30) concluded that the three polystyrene fractions must be considered as different systems. They conclude that a high polymerization temperature produced a branched polymer and a low polymerization temperature a linear one.

An inspection of our data, plotted in Fig. 1, discloses that the subfractions of both the linear and branched polymer fractions of corn starch give curves (for  $\eta_{sp}/C$  versus  $\eta_{sp}$ ) with various slopes. Table III shows the values calculated for  $k$  and the variation in this constant for members (subfractions) of the same major fraction of starch. Therefore, neither of the two *major* fractions is homogeneous in respect to the structure of its components. It should be recalled that the reported data point to an unbranched structure for the simplest member of the linear polymer fraction of corn starch (11, 12, 31). The higher  $[\eta]$  of the fraction soluble in water at 90°C. and the fraction insoluble at 90°C. indicates that they are of a shape different

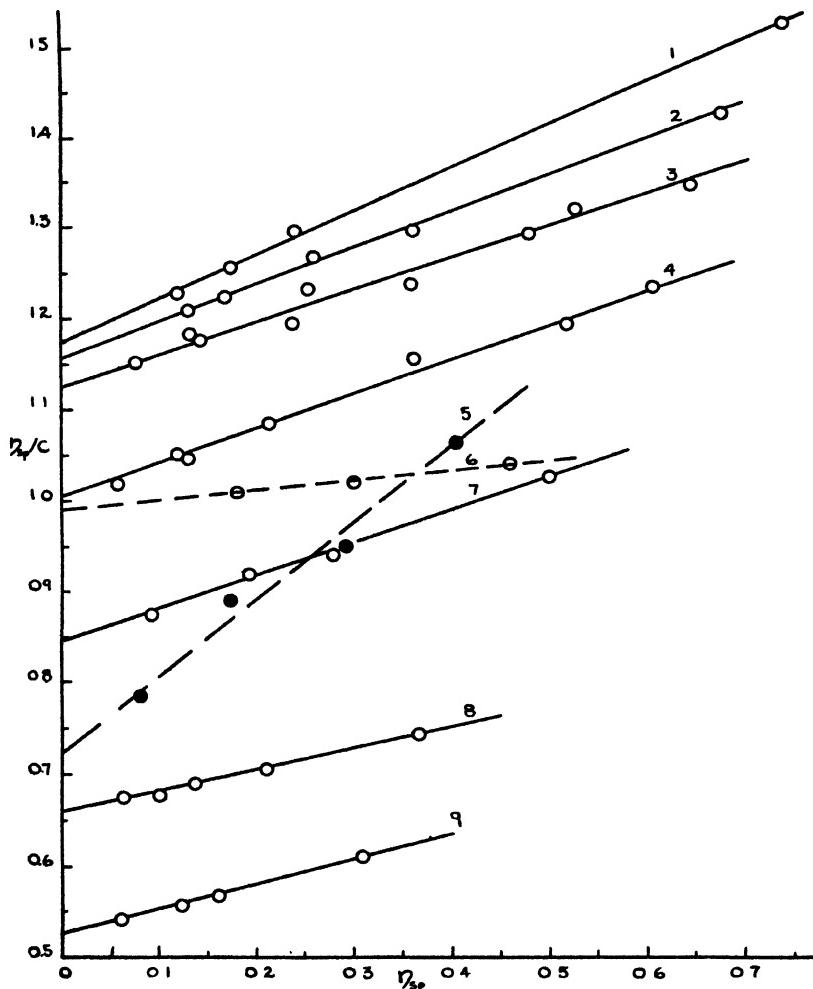


FIG. 1

Viscosities of Solutions of Starch Subfractions and Glycogen in Ethylenediamine

Curve 1, Butanol precipitate of fraction of corn starch, water-soluble at 90°C.; Curve 2, Butanol precipitate of fraction of corn starch, water-insoluble at 90°C.; Curve 3, Potato crystalline amylose; Curve 4, Tapioca crystalline amylose; Curve 5, Corn amylopectin subfraction I (both  $\eta_{sp}/C$  and  $\eta_{sp}$  are divided by 2); Curve 6, Glycogen ( $\eta_{sp}/C$  multiplied by 10); Curve 7, Corn amylopectin subfraction II; Curve 8, Butanol precipitate of fraction of corn starch, water-soluble at 75°C.; Curve 9, Corn amylopectin subfraction III.

The abscissa represents  $\eta_{sp}/C$ ; the ordinate,  $\eta_{sp}$ .

from the 75°C. soluble fraction and, in particular, they possess definitely greater axial ratios. The higher values for  $k$  for the 90°C. soluble and 90°C. insoluble fractions indicates a structural difference when comparison is made with the unbranched 75°C. soluble fraction. One may assume that these differences are explainable on the basis that the unbranched fraction which is soluble in water at 75°C. has altered its shape by assuming the configuration of a helical coil, thus forming a more compact molecule, while the other fractions do not. This and similar conclusions are not consistent with the observation concerning the speed and the completeness with which the 75°C. water-soluble fraction retrogrades, which effect is probably due to a cross-bonding of parallelly oriented linear chains. The 90°C. water-soluble fraction is very much more colloidally stable in water solution. A variation between the fractions which is due to a difference in chemical structure is indicated. It is proposed that the greater axial ratios of the fractions which are soluble in water at high temperatures are due to long linear members in a slightly branched structure.

TABLE III  
*Characteristics of Starch Fractions*

Starch fraction	Intrinsic viscosity	Conversion limit*	Alkali (labile) number†	Iodine titration "linear" material‡	$k\$$
<i>Corn starch</i>				per cent	
<i>Amylopectin</i>					
Subfraction I	1.445	60	3.2	11.8	0.61 ± 0.03
Subfraction II	0.846	55	11.3	8.35	0.40 0.02
Subfraction III	0.526	50-52	13.7	0.00	0.52 0.03
<i>Butanol precipitate</i>					
of 75°C. soluble ext.	0.660	93	35	100.0	0.30 0.04
of 90°C. soluble ext.	1.172		34	100.0	0.41 0.02
from 90°C. insoluble residue	1.158		23	94.0	0.35 0.02
<i>Tapioca</i> crystalline amylose	1.006	89	17	100.0	0.36 0.03
<i>Potato</i> crystalline amylose (Glycogen)	1.125 0.099	97	21	100.0 0.0	0.33 0.02

\* Per cent conversion to Maltose by  $\beta$ -amylase. See text.

† See Ref. (21).

‡ Potentiometric titration with iodine. See text.

$$\$ k = \frac{\eta_{sp}/C - [\eta]}{[\eta]\eta_{sp}}$$

The high value for the percentage of "linear material" (shown in Table III) of the 90°C. water-soluble fraction is not in accord with the view that the viscosity characteristics of this fraction may be attributed to the presence of significant amounts of the branched polymer fraction.

It may also be pointed out that the high viscosities obtained for the amylose subfractions which are soluble only at higher temperature, when compared to that of the 75°C. water-soluble fraction, disagrees with the view advanced by Bates, French, and Rundle (4) that the total amylose fraction of starch is relatively homogeneous in respect to chain length and structure.

The viscosity curve of the end member of the corn amylopectin fraction, subfraction III, and the low  $[\eta]$  obtained is indicative of less asymmetric molecules than exist in any other fraction. Its conversion limit to maltose with  $\beta$ -amylase is also lower than has been observed for any other starch fraction. This result, together with the fact that the absorption of iodine is so low as to indicate a zero content of linear material by the iodine titration test,\* indicates that the structure of this subfraction is highly branched or ramified and the branches are relatively short.

These data may be interpreted otherwise by assuming that amylopectin subfraction III represents the remainder of linear material (from starch) which does not precipitate in the primary separation with butanol of the two major fractions. That is, amylopectin subfraction III, may be supposed to be an assortment of very short linear chains which are not readily crystallizable with butanol, are too short to orient with iodine (4) and are of insufficient length to give a high degree of conversion to maltose with  $\beta$ -amylase. However, this could scarcely be the case since in this event the alkali (labile) number †

\* This subfraction and ones similarly prepared from potato and tapioca starches are the standards of reference (for amylopectin) which have been used by Kerr and Trubell in the spectrophotometric analyses of starches (9).

† The alkali (labile) number (21) of a starch product varies inversely with the molecular size and/or the complexity of its structure. Short unbranched glucopyranose polymers have high alkali (labile) numbers; long chains have lower. Undoubtedly a complexity in the structure, such as a 1-6, $\alpha$ -glucosidic "side linkage" decreases the alkali (labile) number owing to the limited time allowed in the test. For it is known (32) that the degradation by alkali starts at the aldehydic terminal group and continues by enediol splitting and that direct hydrolytic scission of the glucosidic linkages is not involved.

would be considerably greater than 35 (the value found for the unbranched fraction soluble in water at 75°C.) instead of being very much less than this value.

The axial ratios of the amylopectin subfractions II and I, are much higher than that of subfraction III. Indeed, that of subfraction I indicates that the structure is much more extended than that of any of the so-called linear polymer fractions and in this sense it is more linear than the unbranched fraction. The iodine titration values and the higher limit of conversion to maltose with  $\beta$ -amylase indicate the presence of long unbranched members in their branched structures.

These data could not be interpreted by assuming that the results observed are due solely to the effect of unbranched impurities in the amylopectin subfractions I and II. Since, as above stated,  $[\eta]$  is a function of the weight average molecular weight (or "chain length"), a small amount of the relatively short chain, unbranched amylose would have very little effect on the viscosity of a much larger and more extended molecule. Furthermore, the alkali number observed, particularly the extremely low value of 3.26 for amylopectin subfraction I could not be an average\* of two higher values such as would be obtained for example from mixtures of the unbranched polymer with an alkali (labile) number of 35 and amylopectin subfraction III with an alkali (labile) number of 13.7. Indeed, the alkali (labile) number of 3.26 is so extremely low for a starch fraction that the possibility of the presence of any one of the amylose fractions is excluded, at least in an amount which would materially affect the viscosity *per se*.

The alkali (labile) number of amylopectin subfraction II is of a higher order than a subfraction I. Kerr and Severson (11) have shown that cotton readily adsorbs the unbranched fraction of corn starch to the extent of 1% of the weight of the cotton used. Nevertheless, treatment of subfraction II with fourteen times its weight of cotton failed to produce a significant change in the alkali (labile) number, the conversion limit to maltose and the percentage of linear material by iodine titration (see Table II).

It may be noted that the viscosity characteristics of the corn amylopectin subfractions such as the relatively high intrinsic viscosities and the wide spread of the values for  $k$  are not consistent

\* The average alkali number (by weight) for the three amylopectin subfractions is 6.3. This value is in fair agreement with the alkali (labile) number found for the whole fraction which is about 5.7.

with the proposals by Meyer (33) that a highly ramified and relatively symmetrically shaped structure be assigned to amylopectin, nor with his conclusion that only one pattern of branching exists in any particular amylopectin, *e.g.*, corn amylopectin. Our viscometric data for glycogen show the results to be expected from a structure which contains many short branches and which is so highly ramified that notwithstanding its relatively high molecular weight, very low viscosities, which increase very little with the concentration of the solute, are obtained. The ramified structure proposed by Meyer for amylopectin should also result in a compact and non-elongated shape, and obviously this type of structure is difficult to reconcile with the behavior of any of the subfractions of corn amylopectin. The data reviewed suggest that all components of corn starch are relatively asymmetric in shape and that they vary from relatively short, unbranched structures to those which are branched and are of considerable length. In these branched structures are relatively long unbranched sections or members which may contribute to the formation of a highly elongated molecular structure. Intermediate to these two types are those which contain many short branches.

The axial ratio indicated for the amylopectin subfractions could result from one of several different types of branching: (a) the laminated type of branched structure, such as has been proposed by Haworth, Hirst, and Isherwood (34) and by Bawn, Hirst, and Young (35); (b) the type suggested by Staudinger which consists of a long central chain with short regular side branches attached to it; and (c) a branched structure containing long, unbranched members which may be either terminal branches or a terminal portion of a main chain. The higher values for the conversion limit to maltose by  $\beta$ -amylase and the "linear content" by iodine titration of subfractions I and II, compared to those for subfraction III, support the conclusion that the first two subfractions contain longer unbranched members than exist in the structure of the third. Accepting the laminated structure as common to all three, then our data require a shift in the position of the "cross link" (between laminations) to a glucose unit nearer the terminal group as one proceeds from subfraction I to subfraction III. This is shown diagrammatically in Fig. 2. However, it would seem that in this series of branched structures subfraction III would probably have the greatest axial ratio (unless subfraction I is of very great

molecular weight, compared to subfraction III), but this probability is not supported by our viscometric data.

A branched structure which is composed of a long central chain with relatively short side branches would necessarily possess a decreasing length of side branches for the series subfractions I to III to account for the decreasing order of conversion limits to maltose with  $\beta$ -amylase and the percentage of linear material by iodine titration.

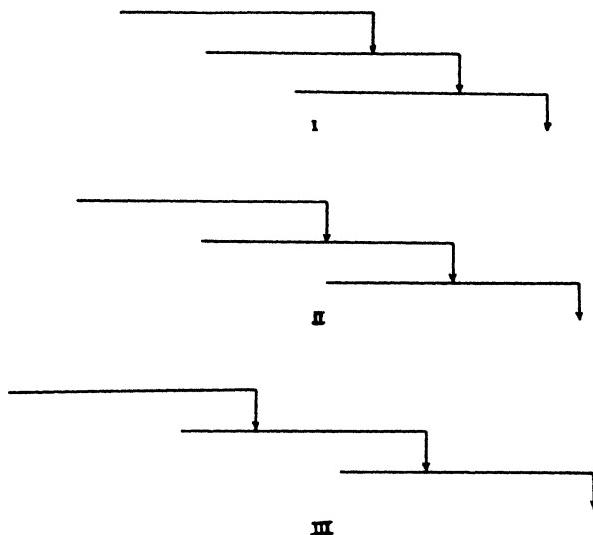


FIG. 2

Laminated Structure for Branched Fraction of Starch which Diagrammatically Shows Possible Variations in Amylopectin Subfractions

However, in this case also, the same difficulty mentioned above is encountered, that subfraction III should have the greatest axial ratio, which it does not.

It seems more reasonable to conclude from our various data that branching is not necessarily according to any definite pattern but that in the subfraction, in addition to whatever other branches may exist, two or more long unbranched members are present. One may contribute to the linearity as indicated by viscosity, iodine reactions, and the differences in the  $\beta$ -amylase conversion limits to maltose; the other may also contribute to the linearity as reflected in viscometric

data but more particularly the differences in the length of this member may explain the variation in the alkali (labile) numbers found for the various subfractions of both major constituents.

That potato and tapioca starches are not simple mixtures of an unbranched type of molecule and one which is highly branched according to a definite pattern, would seem to follow from an examination of the most readily soluble and presumably the most simply constituted fractions of each, the potato and tapioca crystalline amyloses. The viscosity curves for these two amyloses, shown in Fig. 1, indicate that their axial ratios are greater than that of the unbranched fraction of corn starch, and the slopes of their curves are suggestive of a structure which may be intermediate in type\* between unbranched corn amylose and the corn amylose fraction which is insoluble in hot water. This conclusion would seem to follow, especially, for tapioca crystalline amylose for additional reasons: (a) the alkali (labile) number of the tapioca amylose is less than that of either corn or potato crystalline amylose notwithstanding that the potato amylose has the highest axial ratio; (b) the tapioca amylose has the lowest limit of conversion to maltose with  $\beta$ -amylase; and (c) it is the most stable in colloidal solution in water (1% concentration in water at room temperature).

If we assume that the three amyloses differ only in chain length then it follows, in order to explain the definitely greater colloidal instability of the corn fraction, that there is a chain length which is optimum for retrogradation. In this case the tapioca amylose should be less stable in water solution than the potato and should approach the corn amylose in properties. This is not the fact and it seems more reasonable to conclude that, in view of its relative axial ratio, low alkali (labile) value, low conversion limit, and high stability in water solution, the structure of tapioca amylose is branched to some extent.

#### SUMMARY

A method has been given for the preparation of a series of fractions of corn starch. These are essentially subfractions of the so called linear and branched polymer fractions, or of amylose and amylopectin.

\* The viscosities in ethylenediamine, alkali (labile) number, and conversion limit to maltose of tapioca crystalline amylose are of the same order as the respective values for the total fraction of corn starch precipitable by butanol.

A study of the solution viscosity, the alkali (labile) numbers, the conversion limits to maltose by  $\beta$ -amylase, the value for the content of linear material by iodometric methods of these corn starch fractions, and a comparison of these values with those of potato and tapioca starch fractions and of glycogen supports the view that the shape of all starch molecules in selected solvents is rather extended and relatively asymmetric.

If the variations in the asymmetry noted are due to branching in the molecular structures, the data given support the view that the pattern of branching in any one starch is not limited. Some fractions apparently contain molecules with fewer branches than others. In addition, some branches may be considerably longer than others. The data given favor the view that corn starch may be considered as a mixture of unbranched chains and chains which are branched according to several patterns. The crystalline amyloses of potato and particularly tapioca starch may have a small number of branches in their structures.

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LETTER TO THE EDITORS

**The Biological Activity of Oxybiotin for the Rat  
and the Chick**

The synthesis of a hexahydro-2-oxo-1-furo [3, 4] imidazole-4-valeric acid has recently been announced (1). The name oxybiotin has been proposed for this compound because of its high growth-promoting activity for *S. cerevisiae*, *L. casei*, and *L. arabinosus* (2). In a recent note (3), an apparently similar compound has been reported to have biological activity for *S. cerevisiae* and *L. casei*. The present report describes the biotin-like activity of oxybiotin in the rat and the chick.

Thirty male weanling rats of the Sprague-Dawley strain were fed a purified diet (4) containing 10% of egg white throughout the experiment. After seven weeks, growth had ceased, and the typical biotin deficiency symptoms were observed. Two weeks later the animals were divided into six groups of five animals each, and either *d*-biotin, *dl*-oxybiotin, or physiological saline was administered subcutaneously daily for fourteen days as shown in Table I.

TABLE I

Therapy	Weight change in 14 days g.
Saline	- 3
0.1 µg. of <i>d</i> -Biotin	+18
0.2 µg. of <i>d</i> -Biotin	+32
0.2 µg. of <i>dl</i> -Oxybiotin	- 3
0.4 µg. of <i>dl</i> -Oxybiotin	- 2
2.0 µg. of <i>dl</i> -Oxybiotin	+21

After fourteen days of therapy, marked healing had occurred in the groups receiving 0.2 µg. of *d*-biotin or 2.0 µg. of oxybiotin per day. The degree of healing in these groups was similar and superior to that of the group receiving only 0.1 µg. of biotin.

In a prophylactic study, sixty chicks were fed a basal purified diet low in biotin (5). Twenty chicks served as negative controls, and the others, in groups of ten each, were fed the following supplements per 100 g. of ration: (a) 10 µg. of *d*-biotin; (b) 20 µg. of *d*-biotin; (c) 20 µg.

of *dl*-oxybiotin; and (d) 40 µg. of *dl*-oxybiotin. All of the animals receiving either biotin or oxybiotin supplements were completely free from deficiency symptoms throughout the forty day experimental period in marked contrast with the control group. The average weights for these groups at the end of the experiment were as follows: controls, 143 g.; (a) 203 g.; (b) 255 g.; (c) 167 g.; and (d) 158 g.

In a therapeutic study, severe deficiency symptoms were developed by the addition of 10% egg white to the basal diet. After twenty-five days, 4 µg. of *d*-biotin or 8 µg. of *dl*-oxybiotin were administered intramuscularly on alternate days to groups of six chicks each. Three weeks later dermatitis had disappeared from all of the treated chicks. During this interval the control animals gained an average of 51 g., the oxybiotin animals 106 g., and the biotin animals 187 g.

These data demonstrate clearly that oxybiotin exhibits biotin-like activity in both the rat and the chick; oxybiotin is less effective than biotin. It is noteworthy that oxybiotin is the first known compound having activity comparable with that of biotin for higher animals.

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## Book Reviews

**Practical Methods in Biochemistry.** By FREDERICK C. KOCH, Frank P. Hixon Distinguished Service Professor Emeritus of Biochemistry in the University of Chicago; Director of Biochemical Research, Armour and Company, Chicago, and MARTIN E. HANKE, Associate Professor of Biochemistry in the University of Chicago. The Williams and Wilkins Company, Baltimore, Md., 4th Ed., 1943. vii + 353 pp. Price \$2.25.

The following chapters, in the order given, are covered in the present volume: carbohydrates, lipins, proteins and amino acids, nucleoproteins and nucleic acid, hydrogen ion activity and pH, salivary digestion, gastric digestion, intestinal digestion, bile, blood and hemoglobin, the quantitative analysis of blood, the quantitative analysis of urine, the chemical examination of urine for pathological conditions, colorimetric methods for vitamins, chemical tests for hormones, appendix (which includes general laboratory directions and preparation of reagents), and index. The present edition differs from the previous one in that chapters on the colorimetric estimation of vitamins and color reactions of hormones have been added, the chapter on hydrogen ion has been considerably extended to include the fundamental concepts of hydrogen ion activity, ionic strength, and potentiometric methods for determining hydrogen ion activity, some of the newer methods for the quantitative estimation of certain of the amino acids, and better methods for the colorimetric estimation of amino nitrogen and of cholesterol.

There are now a good many laboratory texts and syllabi, both printed and mimeographed, on the market. In essence they include much of the same type of material, both qualitative and quantitative, that is now included in most of the laboratory courses in biochemistry. They differ, however, in the scope of the laboratory work covered, the sequence of experiments, the amount of discussion given, and emphasis on subjects. All attempt to illustrate by means of laboratory experiments a good deal of the lecture material given in a course in biochemistry for medical students and to give the student some practice in the quantitative methods that have proven so useful in clinical laboratories. Few, if any, of the laboratory manuals attempt to introduce the student into the vast realm of biochemical research. Perhaps it is too soon to do this in an introductory laboratory course but it is the writer's opinion that some attempt in this direction should be made in the lectures. Above all and as soon as the necessary foundation has been laid in each of the respective topics covered, students should be required to read a few of the original papers in the field of biochemistry even though this may only serve to acquaint them with the names of biochemical journals and how to look up a subject in the event they desire to obtain more detailed information on any subject covered in the course or to keep abreast with the newer developments.

The present text and most other laboratory manuals assume that students will have had a course in quantitative analysis and in organic chemistry. It has been the

writer's experience that most students appear to be woefully deficient in the technique of preparing and standardizing  $N/10$  solutions of HCl and NaOH and in stoichiometry, let alone the proper background for other quantitative procedures. The term pH is often veiled by a cloud of mystery. It would be well to require students at the beginning of the course to prepare several of the standard solutions, especially the acid and base and to give them additional practice in the choice and use of the proper indicators.

The present volume does not include any material relating to basal metabolism. The important and now generally used technique of estimating serum phosphatase and the methods of determining the filtrable (diffusible) and non-filtrable (non-diffusible) calcium fractions of blood serum as well as the magnesium content of serum and whole blood are not given. Although students are apparently required to determine the chief nitrogenous constituents of their urine after maintaining themselves on a high or a low nitrogen input, no tables giving the nitrogen content of the common foods and the calories yielded to serve as a guide in the selection of the respective diets are given. Too often students carry out these experiments by ingesting large amounts of protein (high protein diet) or of carbohydrate (low protein diet) without any regard of maintaining a fairly constant caloric intake on both diets.

A fairly complete discussion is given of the concepts of activity, activity coefficients, ionic strength, and the Henderson-Hasselbach equation, etc. This phase of biochemistry is usually a stumbling block to students and much effort is required so that students can solve even simple problems. It would have aided students materially if illustrative problems, both solved and for the student to solve, and covering such subjects as simple stoichiometry, pH, paH, buffer mixtures, osmotic pressure, ionic strength, etc., had been included. There is no discussion relating to the accuracy that may be expected in the various quantitative procedures nor is the student taught such simple statistical concepts as the significance of experimental data, probable error, and mean deviation. Too often students will report analytical results with many more figures than have any significance. The biochemical laboratory affords an excellent opportunity of introducing students to the simple concepts of statistical analysis, a subject of which most members of the medical profession are wholly ignorant or unappreciative.

The number of experiments given in the present volume is greater than can be covered in the usual semester's work in biochemistry. This may be considered advantageous since it permits some selection of experiments to suit the needs of particular laboratories.

Although the core of subjects covered in biochemistry in different institutions is essentially the same, the manner of presentation, sequence of subjects, and emphasis placed varies considerably and necessarily so since this is a reflection of the instructors' special interests and their evaluation and personal experience in various phases of the subject. It is impossible during one semester and especially in view of the breadth and scope of biochemistry to teach students all phases of the subject. Nevertheless, the student should at the end of the semester have a due appreciation of the aims and objects of biochemistry, its accomplishments in the elucidation of the body's nutritional needs and the prevention of certain dietary diseases by adequate dietary regimens, the chemical reactions that permit body function, and that this information

as well as future information can only be obtained by the quantitative application of the fundamental concepts of chemistry and physics in adequately controlled experiments. The laboratory then becomes the proving ground not only for diagnostic purposes but also for bringing to light new facts and concepts.

The book is fairly free from errors and misprints. Although the error contained in Van Slyke's original table of amino nitrogen values and carried over into many texts (11° and 732 mm.) has been corrected, a misprint in the 29° and 762 mm. value occurs in the present text.

The fact that Koch and Hanke's book is now in its fourth edition with reprinting of the first and second editions is indicative of its usefulness and general reception. It is to be hoped that in a future edition it will be possible to enlarge its scope considerably and particularly along the lines of the above discussion so as to make it even more useful and generally applicable than it has been in the past.

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**Electrophoresis of Proteins and the Chemistry of Cell Surfaces.** By HAROLD A. ABRAMSON, LAURENCE S. MOYER, and MANUEL H. GORIN; Reinhold Publishing Corporation, New York, 1942. 6 X 9, 341 pages. Price \$6.00.

This book is a cooperative effort of three authors. Abramson is one of the outstanding pioneers in his field. Moyer, who died in the service of this country shortly after publication of the book, accomplished very important and fundamental electrophoretic investigations. And Gorin is well-known for his treatment of various theoretical problems pertaining to the theory of the electrokinetic potential. One was therefore entitled to expect an excellent book written by such a trio. That the book does not fulfill this expectation is probably due primarily to its publication at a time when the manuscript apparently had reached only the first draft and when further revision was probably impeded by war research commenced by some of the authors.

A compromise was attempted between the alternative of writing a new edition to Abramson's fundamental book, *Electrokinetic Phenomena and Their Application to Biology and Medicine* (The Chemical Catalog Company, New York, 1934), with particular emphasis on proteins following the original arrangement of the material or writing a book along entirely different lines. These two divergent tendencies, apparent throughout this book, cause the material to lack a well-organized succession of chapters and sections, result in numerous repetitions, and effect a division of important topics into many scattered sections. Lengthy discussions of details alternate with incomplete treatment of major topics. There are indications that the authors themselves had insufficient time to devote to the final editing and coordination of the figures and tables, leaving this task, as well as that of preparing the subject-index, to persons less qualified. Comparatively minor deficiencies of the book include a few oversights and inaccuracies in the mathematical treatment of theoretical questions—however, less numerous than in Abramson's original book—and the use of undefined terms not familiar to the average chemist and biochemist, particularly in the second half of the book.

There being no better book of similar scope, this book is indispensable to those interested in electrophoresis as applied to biochemical, physiological, biological,

and medical problems, especially when used in combination with Abramson's original book. The present book contains a very complete reference to and review of the enormous number of papers which have appeared in this field. The presentation of material is unbiased in the discussion of controversial questions. The authors have succeeded admirably in impressing the reader with the value of electrophoretic research as documented by the progress made during the last ten years, and in pointing out promising new perspectives. In the latter connection, the authors may well be credited with the merit of having stimulated further progress in this field.

#### DETAILED DISCUSSION

##### *Principal Notations*

The repeated use of nearly the same symbol, such as V and  $V$  or D and  $d$  for different purposes was unnecessary, since the number of symbols is not unusually large. The differences in printing are not sufficiently pronounced to prevent confusion, particularly if the similar symbols are used in succession; for example on page 20. In addition, the meaning of a symbol is not restricted to the definition given in the list. The symbol,  $\phi$ , for example, stands for *fibrinogen* in Table 24, page 184, which is not evident from the legend.

##### *Chapter 1. General Principles of Electric Migration in Liquids*

This introductory chapter touches on all pertinent theoretical and experimental questions, but too much emphasis is placed on details of secondary importance. Five figures of this chapter are imperfect, as are many in subsequent chapters; for example, Fig. 2, which is identical with the  $\zeta - c$  Fig. 72 in Abramson's book, (a) contains numerical concentration data on the abscissa, the units of which are not defined; (b) requires the reading of the legend in order to get the meaning of the ordinate; (c) gives the value 0 instead of the value,  $1 \times 10^{-9}$ . By contrast, Fig. 8 is an excellent aid for the reader.

##### *Chapter 2. Experiments in the 19th Century*

This chapter is a drastically condensed re-edition of the introductory chapter of Abramson's book. There it is used as an excellent introduction, on a historic basis, into the elements of electrophoresis. Here it serves no useful purpose and interrupts the logical sequence of chapters.

##### *Chapter 3. Methods*

Except for the expansions necessary because of progress made, the chapter on methods in Abramson's book is more appealing. The old schematic figure, 12, is replaced by two unnecessarily detailed figures, 13 and 13a, at the expense of other models. Unimportant construction details of the flat cell are discussed at length. The authors endeavored to explain the various modernized moving-boundary methods based on the use of the Schlieren principle. They did not succeed so well, as in the explanation of the microscopic methods.

##### *Chapter 4. Dissolved and Adsorbed Proteins and Related Surfaces*

The discussion of topics begun in Chapter 1 is continued. The comparison between the mobility of protein molecules in solution and of protein molecules adsorbed at surfaces of microscopic particles is very complete and stimulating but somewhat too

detailed. The chapter contains, in addition, a number of heterogeneous sections dealing with the influence on electrophoresis of denaturation, of the size and shape of protein molecules, of surface conductance, and of electro-osmosis. A comparison is made between the mobilities of protein molecules and protein crystals. A few remarks on the mobility of nonproteic substances conclude the chapter.

#### *Chapter 5. Electrokinetic Theory of the Migration of Charged Particles*

Disregarding a few inaccuracies and oversights in the mathematical treatment, this chapter is well written. It would have been desirable to see a few problems discussed in more detail, such as the correlation between electrophoresis and electro-osmosis.

#### *Chapter 6. Electric Mobility and Calculation of the Net Charge*

The procedure is outlined by means of which the net charge of proteins can be calculated from electrophoretic data or from titration curves, provided the sizes and shapes of the protein molecules are known. A number of instructive cases illustrate this significant advance in the interpretation of electrophoretic data. Much and justified emphasis is placed on arguments in favor of Abramson's hypothesis to the effect that the protein mobility at a given ionic strength is proportional to the amount of  $H^+$ —and  $OH^-$ —ions bound by the protein. This chapter, which illustrates well the important contributions made by the authors, contains interspersed topics which are little related to the subject. The chapter closes with a far too brief comparative discussion of the electrokinetic and thermodynamic potential.

#### *Chapter 7. Serum and Plasma Proteins*

This chapter very well demonstrates the importance of the modern moving-boundary methods, e.g., the Tiselius method for the separation, analysis, and comparison of the various proteins present in normal and pathological sera.

#### *Chapter 8. Antibodies, Antigens, and Their Reactions*

Although this may not have been the objective of the authors, this chapter impresses upon the reader the limits of usefulness of the electrophoretic methods in protein research, particularly in the case of specific protein reactions.

#### *Chapter 9. Interaction of Proteins in Mixtures*

Very important recent results, such as the electrophoretic proof of a dissociation and re-association of hemocyanin as a function of pH or the electrophoretic study of the interaction between proteins and proteolytic enzymes, are amply discussed. A better arrangement of the material would have made this chapter most attractive for biochemists.

#### *Chapter 10. Interaction of Proteins at Surfaces*

Having discussed complex biological systems in the preceding chapter, the authors start this chapter with an extensive discussion of comparatively simple systems, thus continuing a topic partly discussed in Chapter 4.

#### *Chapter 11. Enzymes and Hormones*

The authors again take up the discussion of the interaction between proteins and prosthetic groups, begun in Chapter 9, concentrating this time on typical enzymes.

The material is well presented and well organized in contradistinction to the preceding five chapters.

*Chapter 12. Miscellaneous Electrophoretic Investigations of Biological Interest*

This chapter contains a collection of "left-overs." Particular emphasis is placed on discussing the electrophoretic specificity of otherwise apparently identical proteins obtained from different animal classes.

*Chapter 13. Latex*

This chapter represents an appendix to Chapter 10. It contains a review of the interesting work done by Moyer on natural latices of various origin.

*Chapter 14. Surface Chemistry of Cells*

The discussion of the electrophoresis of bacteria begun in Chapter 8 is continued in the first part of this chapter. The second part deals with the electrophoresis of blood cells, and the third part is an extensive review of the work done by Abramson and co-workers on the electric transport of material through the human skin. As in many of the preceding chapters, interesting new perspectives are pointed out and useful working hypotheses are proposed, such as the "key spot theory."

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#### ERRATUM

In ARCHIVES OF BIOCHEMISTRY, Volume 7, No. 1, page 143, the second sentence from the beginning reads as follows:

"When glucose was the carbohydrate no difference could be found in rats fed either fat in the presence of lactose."

The words "in the presence of lactose" should be deleted.

# The Effect of Various Diets Upon the Excretion of Glucuronic Acid by Men, Dogs, Rats, and Rabbits\*

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Received May 17, 1945

## INTRODUCTION

Tollens and Stern (12, 13) observed that the type of diet influenced the quantity of glucuronic acid normally excreted by man, and that the output was generally somewhat higher upon a meat and fat diet than upon a milk and carbohydrate diet. They employed a furfural distillation procedure for determining glucuronic acid in the urine and found quantities ranging from 54 to 653 mg. per 24 hours under varying dietary conditions. Roger and Chiray (9, 10) reported similar observations. Conzen (1), employing Tollens' analytical procedure, found that the normal daily excretion in man ranged from 300 to 590 mg. Maughan, Evelyn, and Browne (5) and Wagreich, Kamin, and Harrow (15), who used the analytical method of Maughan and coworkers, obtained similar results in the case of men. Smaller amounts, ranging from 65 to 306 mg. per 24 hours (mean 164 mg.), were obtained by Deichmann (2) and by Deichmann and Thomas (3) using a modified naphthoresorcinol method.

With the exception of a few observations on frogs (11), rabbits, and dogs, experiments apparently have not been carried out on the effect of the composition of the diet upon the extent to which various compounds, when ingested, are conjugated with glucuronic acid. Mayer (6) was able to show that the administration of glucose was responsible for increasing the quantities of camphor-glucuronate excreted by rabbits deprived of food, while Quick (8) found that a dog kept on a

\* The part of this paper concerned with the effect of various diets on the excretion of glucuronic acid in the rabbit was presented at the 107th semi-annual meeting of the American Chemical Society, Cleveland, April, 1944.

pure carbohydrate diet could synthesize as much as 5 g. glucuronic acid within 24 hours without an increase in nitrogen metabolism.

Stern (14) investigated certain aspects of the effect of fasting upon rabbits and found that the normal output of glucuronic acid became markedly reduced, but when these animals were given an aromatic substance large amounts of conjugated glucuronic acid reappeared in the urine. Makarewitsch-Gaiperin (4), working also with rabbits, and Miller and Conner (7), working with dogs, came to similar conclusions.

This report is concerned with the influence of various types of diet upon the excretion of glucuronic acid by human subjects, rabbits, dogs, and rats. The output of glucuronic acid on the part of animals was determined both before and after the administration of a compound—cyclohexanone—which, when absorbed, leads to the excretion of increased quantities of glucuronic acid in the urine (3).

### EXPERIMENTAL PROCEDURES

The rate of excretion of glucuronic acid was estimated daily for each of three male human subjects and for each of the animals: dogs (female puppies of mixed breeds), albino rabbits, and rats, during periods in which they were fed one of the following diets: a mixed (control) diet, a high protein or peptone diet, a high fat diet, and a high carbohydrate diet. In addition to these observations, the effect of deprivation of food upon the glucuronic acid excretion of dogs, rats, and rabbits, was determined.

After the mean daily excretion of glucuronic acid had been ascertained for each species on each diet over periods of seven to ten days, respectively, each animal was given a daily oral dose of cyclohexanone in addition to a special diet fed again over periods of seven to ten days. Cyclohexanone was given early in the morning by means of a stomach tube and washed down with a small quantity of water. The experimental diet, divided into two doses, was given about three and six hours later.

Each dog and rabbit was kept in an individual metabolism cage and each 24-hour urine specimen was analyzed for its glucuronic acid content. In the case of rats, three animals were kept together in a four-liter pyrex beaker upon a copper screen beneath which there was filter paper. The total excreta for each 24-hour period were analyzed together. (The glucuronic acid content of the feces has been found to be insignificant.) The filter paper saturated with urine, and the feces contaminated by urine, were washed by means of 200 ml. of water into a 500 ml. Erlenmeyer flask and permitted to stand for one hour. Twelve ml. of 19% hydrochloric acid were then added, the total volume was brought to 300 ml. with water, and the mixture heated in a water bath at 75°C. for 45 minutes. After cooling and subsequent filtration through glass wool, the analysis of aliquot samples was continued as indicated in our method (2). Like that of Maughan, Evelyn, and Browne, this procedure employs urine without the separation or removal of interfering constituents and makes use of Tollens' naphthoresorcinol reagent. It differs from theirs, however, in that it makes use of (1) a preliminary hydrolysis at 75°C., (2) a great excess of naphthoresorcinol, and (3)

heating the hydrochloric acid-naphthoresorcinol mixture at 50°C. These modifications of Tollens' method make it possible to determine quantities up to 320  $\mu\text{g}$ . of free or conjugated glucuronic acid per sample (with an error of  $\pm 5\%$ ) in the presence of the common constituents of the urine of laboratory animals and men.

### RESULTS AND DISCUSSION

The mean quantities of glucuronic acid excreted per day, by human subjects during a series of seven-day periods characterized for experimental purposes by the ingestion of a control (mixed) diet, a high protein diet, a high fat diet, and a high carbohydrate diet, were, respectively, 199, 250, 191, and 171 mg. The output of glucuronic acid by human subjects is significantly higher upon a protein than upon a mixed, high carbohydrate, or high fat diet. The quantity of glucuronic acid excreted under any of these conditions is however only about half of that reported by other investigators (1, 5, 9, 12) (Table I).

TABLE I  
*The Experimental Diets for Three Male Subjects and Their Mean Daily Output of Glucuronic Acid*

Composition of daily diet	Mean 24 hour output of glucuronic acid per man mg.	Probable error	Total num- ber of 24 hour samples analyzed
<i>Mixed (control) diet</i> (Restricted only in regard to ingestion of foods likely to contain artificial flavors, colors, or preservatives.)	199.0	$\pm 6.6$	20
<i>High protein diet</i>	250.0	$\pm 5.7$	16
800 g. lean meat			
800 g. lean fish			
150 g. of chicken egg			
800 g. skimmed milk			
400 g. cottage cheese			
<i>High fat diet</i>	191.4	$\pm 5.5$	11
450 g. of 30% cow's cream			
2000 g. whole milk			
<i>High carbohydrate diet</i>	171.0	$\pm 2.7$	17
300 g. spaghetti			
400 g. potatoes			
800 g. skimmed milk			
200 g. bread			
100 g. sirup			

TABLE II

*The Experimental Diets for (45) Rabbits and Their Mean Daily Output of Glucuronic Acid*

Composition of daily diet	Mean 24 hour output of glucuronic acid per rabbit <i>mg.</i>	Prob- able error	Total number of 24 hour samples analyzed	Mean 24 hour output of glucuronic acid per rabbit given daily one oral dose of 0.3 ml. of cyclo- hexanone* <i>mg.</i>	Prob- able error	Total number of 24 hour samples analyzed
<i>Mixed (control) diet</i> Pratt's Complete Rabbit Pellets <i>ad libitum</i>	33.4	±2.5	58	260.6	±1.1	46
<i>High peptone diet</i> 30 g. of "Bacto-peptone" (25% aqueous solution) and about 50 g. of Pratt's Complete Rabbit Pellets	25.2	±2.0	37	300.0	±1.9	32
<i>High fat diet</i> 50 g. of 30% cow's cream and about 50 g. of Pratt's Complete Rabbit Pellets	40.7	±1.9	44	242.6	±1.6	33
<i>High carbohydrate diet</i> 30 g. of an equal mixture of soluble starch and anhydrous dextrose (25% aqueous suspension) and about 50 g. of Pratt's Complete Rabbit Pellets	39.3	±2.7	31	180.9	±1.4	30
<i>Vegetable diet</i> 400 g. spinach 700 g. lettuce 150 g. carrots	32.9	±2.7	48	163.2	±1.1	37
Fasting	17.8	±1.2	16	130.5	±1.1	46

\* The approximate single lethal oral dose of cyclohexanone for rabbits is 2.0 ml./kg.

TABLE III  
*The Experimental Diets for (24) Dogs and Their Mean Daily Output of Glucuronic Acid*

Composition of daily diet	Mean 24 hour output of glucuronic acid per dog mg.	Probable error	Total number of 24 hour samples analyzed	Mean 24 hour output of glucuronic acid per dog given daily one oral dose of 0.4 ml. of cyclohexanone mg.	Probable error	Total number of 24 hour samples analyzed
<i>Mixed (control) diet</i> 250 g. lean meat 50 g. ox tail 75 g. vegetables	15.8	±1.2	44	303.5	±22.7	14
<i>Peptone diet</i> 70 g. "Bacto-peptone" (25% aqueous solution)	16.2	±1.9	15	315.9	±27.7	11
<i>High fat diet</i> 100 g. of 30% cow's cream	11.8	±0.7	16	272.6	±17.8	13
<i>Carbohydrate diet</i> 70 g. of equal mixture of soluble starch and anhydrous dextrose (25% aqueous suspension)	11.2	±1.3	16	325.0	±13.4	12
Fasting	17.8	±1.2	16	287.5	±24.5	6

Rabbits excreted about 33 mg. and dogs about 15 mg. of glucuronic acid per day. The individual diets caused no statistically significant fluctuation from these figures (Tables II and III). Rats excreted about 1.5 mg. of glucuronic acid per day when fed a mixed, a high fat, or a high peptone diet. However, the mean daily output was significantly lower when the animals were given the high carbohydrate diet (Table IV). (When rats were fed Purina Fox Chow Pellets, the mean daily output of glucuronic acid was about 6 mg. The reason for this high rate of excretion is not apparent, but it is possible that the pellets contained some ingredient that is not a common constituent of this food.)

TABLE IV

*The Experimental Diets for (134) Rats and Their Mean Daily Output of Glucuronic Acid*

Composition of daily diet	Mean 24 hour output of glucuronic acid per rat mg.	Prob- able error	Total number of 24 hour samples analyzed	Mean 24 hour output of glucuronic acid per rat given daily one oral dose of 0.08 ml. of cyclo- hexanone* mg.	Prob- able error	Total number of 24 hour samples analyzed
<i>Mixed (control) diet</i> Biscuits <i>ad libitum</i> con- taining 25 g. corn starch, 41 g. casein, 17 g. lard, 5 g. cod liver oil, 7 g. yeast, and 6 g. of a salt mixture, (Salt mixture contains 8 g. NaCl, 9 g. MgSO <sub>4</sub> , 13 g. NaH <sub>2</sub> PO <sub>4</sub> , 29 g. K <sub>2</sub> HPO <sub>4</sub> , 40 g. Ca <sub>2</sub> H <sub>7</sub> - (PO <sub>4</sub> ) <sub>2</sub> ·H <sub>2</sub> O, 0.9 g. iron citrate and 0.1 g. KI.)	1.5	±0.1	54	26.5	±0.7	18
<i>Peptone diet</i> 6 g. "Bacto-peptone" (40% aqueous solution)	1.5	±0.1	33	27.3	±2.2	18
<i>High fat diet</i> 15 g. of 30% cow's cream	1.5	±0.1	48	36.7	±5.8	51
<i>Carbohydrate diet</i> 6 g. of equal mixture of soluble starch and an- hydrous dextrose (40% aqueous suspension)	0.6	±0.06	51	32.5	±4.4	18
Fasting	1.5	±0.1	51	25.0	±2.4	60

\* The single lethal oral dose (LD<sub>50</sub>) of cyclohexanone for rats is 1.84 ml./kg.

Deprivation of food (for seven to ten days) brought an unexpected response on the part of dogs and rats. The output of glucuronic acid remained just as high as it had been when the animals were given the

various diets (Tables III and IV). The rabbits responded differently in that the rate of the excretion of glucuronic acid decreased strikingly, in conformity with the previous results of Stern (14) (Table II). In order to determine whether this behavior characterized the herbivorous animals as a group, in contradistinction to carnivora or omnivora, the rate of glucuronic acid excretion by guinea pigs was followed over a period of nine days, while three of them were being fed a mixture of oats, corn, and Pratt's Pellets, and the other three were deprived of food. The excretion of glucuronic acid was found to be very nearly the same in both groups, approximating 3.5 mg. per pig per day. It is therefore apparent that some physiological peculiarity on the part of the rabbit differentiates this animal from the other species employed.

In another group of experiments, the capacity of the same animal species (under similarly varied dietary conditions) to bring about the conjugation of sublethal doses of cyclohexanone with glucuronic acid was measured. In the case of dogs and rats, the type of diet fed during the administration of cyclohexanone did not (statistically) significantly influence the outcome (Tables III and IV). The rabbits were again in a class by themselves, in that their ability to excrete the compound in conjugation with glucuronic acid was great while they were taking a high peptone, a mixed, or a high fat diet, and was lessened while they were fed carbohydrates or vegetables (Table II).

During the period of food deprivation (six days), the animals again gave an unanticipated response. The discontinuance of the regular intake of (mixed) food had no effect upon the conjugation of cyclohexanone with glucuronic acid in the case of dogs and rats (Tables III and IV). The quantities of cyclohexanone excreted in conjugation with glucuronic acid by fasting rabbits decreased by half as compared with those found while the animals were taking a mixed diet (Table II).

### CONCLUSIONS

(1) While taking mixed (control) diets, human subjects, rabbits, dogs, and albino rats excrete the following mean daily quantities of glucuronic acid: 199, 33, 15, and 1.5 mg., respectively. The various diets used in feeding experiments (mixed, high protein or peptone, high fat, high carbohydrate) produced no statistically significant differences in the extent of excretion of glucuronic acid by either human subjects, rabbits, dogs, or rats with the exception of (a) an increased

excretion by human subjects ingesting a high protein diet, and (b) a diminished excretion by rats given a carbohydrate diet.

(2) The various diets (mixed, pure peptone, almost pure fat, or pure carbohydrate) induced no statistically significant variation in the output of glucuronic acid when dogs and rats were tested in regard to their ability to conjugate sublethal doses of cyclohexanone. In rabbits, the type of diet ingested is important. The quantity of glucuronic acid excreted in the urine remained at nearly the same level when a mixed, a high peptone, or a high fat diet was given, but a diet high in carbohydrate content and a vegetable diet each induced a significantly decreased output.

(3) In dogs and rats, fasting (six to ten days) did not significantly influence (a) the quantity of glucuronic acid normally excreted, nor (b) the animal's ability to conjugate cyclohexanone with glucuronic acid. Eight to twelve days of fasting induced in rabbits (a) a marked drop in the output of glucuronic acid, and (b) a marked lowering in the animal's ability to conjugate cyclohexanone with glucuronic acid.

(4) The glucuronic acid excreted in the urine of healthy and untreated dogs and rats appears to be entirely of endogenous origin; the bulk of the glucuronic acid excreted by rabbits appears to be of exogenous origin.

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# **Effect of Liver Injury Upon the Excretion of Glucuronates and Organic Sulfates**

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Received May 17, 1945

## **INTRODUCTION**

In the belief that the liver is the organ chiefly responsible for the conjugation of certain organic compounds with glucuronic acid, a number of investigators have regarded the rate of excretion of glucuronates, following the administration of some compound so excreted in the urine, as a measure of the functional capacity of the liver (1, 5, 8, 9, 10, 11). Others have found no consistent relationship between liver injury and output of glucuronic acid (4, 6, 7, 13, 14). Apparently the rate of excretion of an organic compound as organic sulfate has not been considered as a measure of liver function.

This paper reports observations on the extent to which poisoning, either by yellow phosphorus or by a mixture of chloroform and carbon tetrachloride, affects the ability of rabbits to conjugate cyclohexanone with glucuronic acid and benzene with sulfuric acid.

## **EXPERIMENTAL PROCEDURES**

Albino rabbits, weighing from 2.4 to 2.6 kg., were purchased from a local breeder and kept under observation for at least two weeks before any experimental procedure was undertaken. They had free access to Purina Rabbit Pellets and to water. Liver injury was induced in each of 20 animals by oral administration of 2 to 4 doses of yellow phosphorus, each dose being 3 mg./kg. body weight. The compound was given twice or three times per week as a 0.25% solution in olive oil. In each of a second group of (21) rabbits liver injury was induced in a similar manner with an equal mixture of chloroform and carbon tetrachloride (2 to 5 doses, each dose 0.2 ml./kg.). Twenty-four hours after the last administration of one or the other of these preparations, each of ten of the rabbits poisoned with yellow phosphorus and each of twelve

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An abstract of this paper has appeared in the *Federation Proceedings*, Vol. 4, No. 1, page 116 (1945).

TABLE I

*Excretion of Glucuronic Acid\* in the Urine of Normal Rabbits and Rabbits Suffering from Liver Injury Induced by Repeated Oral Administration of Yellow Phosphorus or a Mixture of Equal Volumes of Chloroform and Carbon Tetrachloride*

Identification number of rabbit	Number of doses of liver poison given	Degree of liver injury produced	Glucuronic acid output in urine per 24 hours mg.
<i>Normal</i>			
D 8924	0	none	410
D 8723	0	none	528
D 8925	0	none	536
D 9617	0	none	592
D 8923	0	none	697
D 9616	0	none	775
D 8374	0	none	840
D 9615	0	none	856
D 8338	0	none	1190
			Mean 705
<i>Liver Injured by Yellow Phosphorus†</i>			
D 367	3	severe	372
D 369	3	severe	478
D 370	3	severe	483
D 368	3	severe	512
D 433	4	severe	667
D 366	3	severe	765
D 434	4	moderate	1018
D 444	4	severe	1045
D 545	2	severe	1045
D 441	4	moderate	1152
			Mean 750
<i>Liver Injured by Chloroform -Carbon Tetrachloride‡</i>			
D 8316	3	moderate	217
D 8315	5	moderate	290
D 8314	3	moderate	500
D 8352	2	moderate	530
D 8351	2	moderate	611
D 8349	5	moderate	653
D 9706	4	moderate	839
D 9708	4	moderate	909
D 9585	2	moderate	922
D 9582	2	severe	962
D 8350	3	moderate	1026
D 9713	4	moderate	1056
			Mean 725

\* A single oral dose of 0.8 ml. of cyclohexanone was given to stimulate excretion of glucuronic acid.

† Each dose given was equal to 3 mg./kg.

‡ Each dose given was equal to 0.2 ml./kg.

(The approximate single lethal oral doses are per kilogram of rabbit: cyclohexanone, 2.0 ml.; yellow phosphorus (0.25% in olive oil), 5.0 mg.; mixture of equal volumes of chloroform and carbon tetrachloride, 0.94 ml.)

TABLE II

*Percentage Relationship of Organic to Total Sulfates\* in the Urine of Normal Rabbits and Rabbits Suffering from Liver Injury Induced by Repeated Oral Administration of Yellow Phosphorus or a Mixture of Equal Volumes of Chloroform and Carbon Tetrachloride*

Identification number of rabbit	Number of doses of liver poison given	Degree of liver injury produced	Percentage relationship of organic to total sulfates in urine per 24 hours
<i>Normal</i>			
D 9646	0	none	93
D 9645	0	none	90
D 8955	0	none	86
D 8140	0	none	85
D 8141	0	none	80
D 9647	0	none	76
D 9648	0	none	69
D 9644	0	none	68
D 8954	0	none	48
D 8956	0	none	48
			Mean 75.5
<i>Liver Injured by Yellow Phosphorus†</i>			
D 563	2	severe	92
D 561	2	moderate	82
D 559	2	moderate	79
D 543	2	severe	75
D 562	2	severe	71
D 560	2	severe	58
D 544	2	severe	44
D 542	2	severe	41
D 483	3	moderate	40
D 481	3	severe	30
			Mean 62.0
<i>Liver Injured by Chloroform—Carbon Tetrachloride‡</i>			
D 9673	2	moderate	65
D 9671	2	mild	63
D 8976	3	moderate	48
D 8974	3	moderate	38
D 8251	4	severe	38
D 8977	3	moderate	31
D 8975	3	moderate	30
D 8252	4	severe	22
D 8253	4	moderate	0
			Mean 36.4

\* A single oral dose of 1.2 ml. of benzene was given to stimulate excretion of organic sulfates.

† Each dose given was equal to 3 mg./kg.

‡ Each dose given was equal to 0.2 ml./kg.

(The approximate single lethal oral dose of benzene is, for rabbits, 3.2 ml./kg. body weight.)

of the animals poisoned with chloroform and carbon tetrachloride was given a single oral dose of cyclohexanone (0.8 ml. per animal) to stimulate excretion of conjugated glucuronic acid, while each of the remaining rabbits was given a single oral dose of benzene (1.2 ml. per animal) to stimulate excretion of organic sulfates (2). Similar doses of cyclohexanone or benzene were given to other unpoisoned (control) rabbits. The quantities of glucuronic acid or sulfates excreted in the urine of each rabbit were determined by methods described elsewhere (3, 12). (The analytical method employed for the estimation of sulfates determined "total" and "inorganic" sulfates chemically and "organic" sulfates by difference.) All rabbits were killed by air embolism, and the liver of each animal was removed and examined for gross and microscopic evidences of abnormality.

Tables I and II present the observations made.

### RESULTS AND CONCLUSIONS

Moderate or severe injury of the liver as indicated by the characteristic lesions of poisoning by phosphorus or by the mixture of carbon tetrachloride and chloroform was found in the tissues of all experimental animals. No gross or microscopic evidence of injury was found in the tissues of the control animals.

The conjugation and excretion of cyclohexanone with glucuronic acid was not impaired in rabbits suffering from moderate to severe injury of the liver induced by the repeated oral administration of sub-lethal doses of yellow phosphorus or by a mixture of chloroform and carbon tetrachloride administered in a similar manner.

The rate of excretion of benzene as the organic sulfate became significantly impaired in rabbits poisoned by the mixture of chloroform and carbon tetrachloride. Conjugation and excretion of benzene as the organic sulfate was slightly impaired in rabbits poisoned by yellow phosphorus, but the changes induced were statistically insignificant.

The observations made do not support the thesis that the rate of excretion of glucuronic acid is indicative of the functional integrity of the liver.

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# The Fungistatic and Fungicidal Action of Fatty Acids and Related Compounds

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Received May 25, 1945

## INTRODUCTION

The current interest in dermatomycoses has suggested further investigation of the reported activity of fatty acids and related compounds as antimycotic agents. This problem was first studied by Clark (1) in 1899 who reported the effect of acids, including fatty acids, on the germination of fungi. The systematic investigation of Kiesel (2) in the Pasteur Institute in 1913 revealed many of the facts known today about the characteristics of the antimycotic action of fatty acids. He found that (a) the activity of saturated fatty acids increases as the number of carbon atoms in the fatty acid chain increases up to 11 carbon atoms, (b) the branched chain fatty acids are less active than those with straight chains and an equal number of carbon atoms and (c) substitution of hydrogen by hydroxyl decreases activity.

Japanese workers (3, 4, 5) in 1931 and 1933 essentially confirmed Kiesel's work but found that with the saprophytic wood-rotting fungi the optimum activity was with the 12 carbon acid. They also investigated the effect of hydrogen ion concentration and found fatty acid anions to be inactive. Unsaturated acids were found to be more active than the corresponding saturated acids and dicarboxylic acids were almost without activity. Kirby *et al.* (6), Peck *et al.* (7, 8), Baechler (9), Hoffman *et al.* (10, 11) and Rigler and Greathouse (12) confirmed the findings of earlier workers and extended them to other conditions and other organisms. Cowles (13) showed that at low pH values fatty acids have a bactericidal action and that their activity increases with chain length. Keeney *et al.* (14, 15) reported fungicidal activity for the long chain carbon acids but much less or no killing action by the short chain members of the fatty acid series.

Our experiments were designed to measure the fungistatic and fungicidal actions of fatty acids and related compounds and to study the conditions and chemical structure which give maximum activity.

## METHODS

*Testing Procedure.* Two methods were employed for the static experiments. (a) Submerged cultures in dextrose-nutrient broth containing the desired concentrations of antimycotic agents and adjusted to the pH value under study were agitated for 48 hours for fast growing organisms and 7 days for slow growing species. (b) The active ingredient was added to dextrose-nutrient agar buffered at the pH under study and the inoculum spread over the surface of the hardened plates. In both of these procedures the medium was buffered with 0.02 M phosphate or citrate, the dextrose added after sterilization, and pH measurements made with a glass electrode immediately before use. The inoculum was prepared from spores and/or vegetative elements of mature cultures and added to the plates or tubes in 0.05 ml. quantities. The active ingredients were prepared in 10 or 20% stock solutions in 70% ethanol, the pH adjusted so that the desired value would be obtained upon dilution and the solutions sterilized by minimal heating. From a series of closely spaced concentration levels, that concentration which would just inhibit growth was determined by interpolation from the limiting values giving growth and no growth.

To study the fungicidal action, test solutions of fatty acids were made up in 0.02 M phosphate buffer and adjusted to the desired pH. Spore suspensions were made from mature cultures of the fungi grown on Sabouraud agar plates. These were suspended in water by vigorous mechanical agitation and entrapped gas bubbles removed by repeated application of a vacuum to the spore suspension. The spores were filtered through cotton to remove the mycelial elements. The *Trichophyton* spores were obtained from plates 10-14 days old and *Aspergillus* spores from 4-7 day old cultures. At zero time about 100,000 spores per ml. were inoculated into the equilibrated fungicidal solutions and at the indicated time intervals 1 ml. was removed to centrifuge tubes containing 10 ml. of sterile 1% NaHCO<sub>3</sub>. The tubes were centrifuged for three minutes in a Fisher Anglehead Safety Centrifuge and the supernatant decanted. Fifteen ml. of Sabouraud's agar at 45°C. were added to each tube and, after agitating by rubbing the inside walls of the centrifuge tubes with a pipette, the agar was poured into Petri plates. The surviving spores were estimated after incubation for sufficient time to permit good growth in the controls. The method was tested for recovery of spores and for neutralization of static action and found to be satisfactory.

The cultures employed are listed in Table III.

*Preparation of Compounds.* The saturated fatty acids used in this study were obtained from commercial sources.

Undecylenic acid,<sup>1</sup> obtained from Baker Castor Oil Company, was purified by

<sup>1</sup> The term "Undecylenic Acid" used throughout this paper refers to the common 11-carbon unsaturated acid obtained from the thermal decomposition of castor oil. It has appeared in the literature as 10-undecylenic acid, 10-undecenoic acid, 10-hen-decenoic acid and *i*-hen-decenoic acid.

recrystallization of its zinc salt from dioxane. The free acid liberated from purified zinc undecylenate distilled at 130°C. at 1 mm. pressure; acid number 303 (theory 304.4); m.p. 22°;  $n_D^{25} = 1.4472$ . Ethyl undecylenate, undecylenyl alcohol, undecylenoylamide and undecylenyl acetate were prepared from purified undecylenic acid using conventional methods. N-hydroxyethyl undecylenoylamide was obtained by the interaction of undecylenoyl chloride with excess ethanolamine. This compound, purified by recrystallization from water, melted at 68° (all melting points are uncorrected).

2-Hendecenoic acid was prepared according to the method of Crossley and Le Sueur (16) from hendecanoic acid. 5-Hexenoic acid was obtained in low yield from  $\epsilon$ -amino-caproic acid by the method of Wallach (17). 3-Decenoic acid was prepared from n-heptaldehyde and succinic acid through the intermediate n-hexylparaconic acid by the method of Fittig (18).

A number of  $\beta$ -substituted acrylic acids prepared for this study are reported in the literature. These compounds have been prepared usually by some modification of the Perkin reaction. In this study the Doebner (19) modification of the Perkin reaction was found to give more satisfactory results than the other modifications investigated. 2-Hexenoic acid (20), 2-nonenoic acid (21), 2-dodecenoic acid (22), 2,4-hexadienoic acid (19), 5-phenyl-2-pentenoic acid (23) and 5-phenyl-2,4-pentadienoic acid (19) were prepared by this method from n-butylaldehyde, n-heptaldehyde, n-decaldehyde, crotonaldehyde, hydrocinnamaldehyde and cinnamaldehyde respectively. The details of the method are essentially those given by Johnson (24) for the preparation of 2-nonenoic acid.

Additional  $\beta$ -substituted acrylic acids, previously unreported in the literature, synthesized for this study were 4-ethyl-2-hexenoic acid: b.p. 107°/3 mm., neut. equiv. 141 (calc'd 142), amide m.p. 104–105°; 4-ethyl-2-octenoic acid: b.p. 120°/3 mm., neut. equiv. 170 (calc'd 170), amide m.p. 97–97.5°; 2,12-tridecadienoic acid: b.p. 163°/1 mm., neut. equiv. 211 (calc'd 210), amide m.p. 109–110°; and 2-tetradecenoic acid: b.p. 160°/2.5 mm., neut. equiv. 230 (calc'd 226), amide m.p. 114–115°. These compounds were prepared by the malonic acid-pyridine condensation from 2-ethylbutyraldehyde, 2-ethylhexaldehyde, undecylenaldehyde and n-dodecaldehyde respectively. These aldehydes were obtained from commercial sources.

5-Cyclohexyl-2-pentenoic acid: m.p. 64–66°, neut. equiv. 184 (calc'd 182), amide m.p. 178–179°, was synthesized from  $\beta$ -cyclohexylpropionaldehyde (25) by the malonic acid condensation. The aldehyde was obtained by the reaction of cyclohexylethyl magnesium bromide with ethyl orthoformate (26).

5-Phenylvaleric acid (27), 5-cyclohexylvaleric acid (28) and 4-ethyl-octanoic acid (29) were obtained by low pressure hydrogenation of the corresponding unsaturated acid using Adams' catalyst. Nitration of 5-

phenylvaleric acid followed by catalytic reduction of the mixed nitro isomers in ethyl acetate solution gave a mixture of *o*- and *p*-aminophenylvaleric acids. These were separated by fractional crystallization of the acetyl derivatives from water. 5-(*p*-Aminophenyl)valeric acid (30) was converted to 5-(*p*-hydroxyphenyl)valeric acid by reacting with nitrous acid. The hydroxy compound melted at 118–120° and had a neut. equiv. of 193.5 (calc'd 194).

Undecenoyl thiourea, m.p. 123.5–124.5°, and S-undecenoyl thioruronium chloride, m.p. 120.5–121.5°, were obtained by the method of Dixon and Taylor (31). n-Heptylmercaptoacetic acid, m.p. 40°, neut. equiv. 192 (calc'd. 190), was prepared by the reaction of n-heptyl bromide with the sodium derivative of ethyl thioglycollate followed by hydrolysis of the condensation product. 11,11'-Dithiodihendecanoic acid, m.p. 81.5–82.5°, was obtained by the reaction of 11-iodohendecanoic acid with sodium disulfide. Reduction of the dithio derivative gave the corresponding mercapto derivative, 11-thiolhendecanoic acid, m.p. 84–85°. Other sulfur containing compounds used in this study were supplied by Prof. E. Emmet Reid of Johns Hopkins University (32).

## RESULTS

### *Static Action*

The concentrations of antimycotic agent necessary to inhibit growth by the submerged culture method agreed very closely with those obtained by the surface growth method. The tables give the averages obtained from 2 to 4 experiments. In agreement with the findings of previous investigators the data summarized in Table I show that (a) the activity of fatty acids increases with chain length, (b) the unsaturated acids are slightly more active than the saturated acids and (c) there is no special merit in fatty acids containing an odd number of carbon atoms over the naturally occurring even numbered homologues. Our data show, however, that the optimum chain length is apparently determined by the resistance of the organisms and the solubility of the fatty acids in question. Thus, for *A. niger* the optimum chain length is 11 carbon atoms but for the more sensitive *T. interdigitale* the 13-carbon acids are most active. *T. purpureum* is even less resistant and is inhibited by the 14-carbon acids, suggesting that the longer chain compounds fail to show antimycotic effects because, due to lack of solubility, a static concentration cannot be obtained. One might

TABLE I  
*Static Action of Fatty Acids in Dextrose Nutrient Medium at pH 6.5*

Acid	C Atoms	Inhibiting concentrations per cent				<i>T. purpureum</i>
		<i>A. niger</i>	<i>S. aureus</i>	<i>T. interdigitale</i>		
Palmitic	16	>.08	>.08	>.08	>.08	>.08
Myristic	14	>.08	>.08	>.08	>.08	.001
2-Tetradecenoic	14*	>.04	.007	.008	.008	.001
Tridecanoic	13	>.08	.07	.003	.003	.0015
Lauric	12	>.08	.05	.0035	.0035	.002
2-Dodecenoic	12*	>.08	>.08	.003	.003	.0015
Hendecanoic	11	.045	.02	.005	.005	.003
Undecylenic	11*	.04	.02	.004	.004	.002
Capric	10	.06	.015	.0075	.0075	.005
Pelargonic	9	.07	.04	.01	.01	.007
2-Nonenoic	9*	.06	.03	.007	.007	.005
Caprylic	8	.15	.06	.015	.015	.009
Heptylic	7	.3	.15	.03	.03	.02
Caproic	6	.5	.5	.15	.15	.09
2-Hexenoic	6*	.25	.15	.15	.15	.08
Propionic	3	3.0	.75	.5	.5	.3

\* Unsaturated acids.

conclude, for example, that *A. niger* would be inhibited by about .03% lauric acid but, since lauric acid does not dissolve to that extent, no inhibition results; it does dissolve to the extent of .0035% which is sufficient to inhibit *T. interdigitale*. There appears to be no evidence of

TABLE II  
*Effect of pH on the Fungistatic Action of Fatty Acids*

Acid	Inhibiting concentrations per cent				<i>T. interdigitale</i>			
	pH 4.5	5.5	6.5	7.5	pH 4.5	5.5	6.5	7.5
Palmitic	>.1	>.1	>.1	>.1	>.1	>.1	>.05	.03
Myristic	>.1	>.1	>.1	>.1	>.05	>.05	>.05	.005
Tridecanoic	>.1	>.1	>.1	>.1	.015	.001	.003	.005
Lauric	>.1	>.1	>.1	.07	.0005	.0015	.0035	.005
Hendecanoic	>.15	.02	.045	.08	.001	.002	.005	.008
Undecylenic	.015	.02	.04	.07	.006	.001	.004	.005
Capric	>.1	.03	.06	.2	.001	.004	.0075	.01
2-Nonenoic	.02	.05	.07	.3	.002	.006	.01	.02
Caprylic	.03	.09	.15	.4	.003	.009	.015	.03
Heptylic	.05	.1	.3	.45	.007	.01	.03	.1
Caproic	.06	.2	.5	1.5	.008	.02	.15	.7
Propionic	.1	.3	2.0	5.0	.02	.07	.5	1.5

specificity for any of the four organisms since the resistance curves follow each other in a regular manner.

The data in Table II show that the fatty acids increase in activity with decreasing pH provided the low pH values do not make the compound so insoluble that a static concentration for the organism under test cannot be obtained. The change in activity with hydrogen ion concentration is much greater for the short chain acids, suggesting that the ion of the long chain compound—or some aggregate or micellar form of it which may exist in solution—exerts additional action. A similar assumption might explain the inhibition sometimes observed with emulsions of the long chain fatty acids when used in concentrations in excess of their solubility.

Undecylenic acid was chosen as being representative of the more active members of this type of compound and the amount required for stasis of a number of organisms is recorded in Table III. It is evident that in this respect the *T. interdigitale* strain is representative of the

TABLE III  
*Fungistatic and Bacteriostatic Spectrum of Undecylenic Acid at pH 6.5*

Organism	Source	Inhibiting concentration per cent
<i>Trichophyton interdigitale</i>	C. W. Emmons No. 598	.004
<i>Trichophyton purpureum</i>	A.T.C.C. No. 7203	.0015
<i>Trichophyton violaceum</i>	A.T.C.C. No. 7205	.0025
<i>Microsporum audouini</i>	A.T.C.C. No. 9082	.0025
<i>Epidermophyton inguinale</i>	N. Y. Skin and Cancer Hospital	.0025
<i>Torula</i> sp.	From fatal case of <i>Torula</i> Meningitis—Post Graduate Hospital, New York	.015
<i>Saccharomyces cerevisiae</i>	Laboratory isolate	.01
<i>Monilia albicans</i>	A.T.C.C. No. 2112	.007
<i>Aspergillus niger</i>	Thom and Church No. 167	.05
<i>Penicillium notatum</i>	N.R.R.L. No. 832	.045
<i>Neurospora crassa</i>	Beadle's Parent Strain	.03
<i>Mycobacterium tuberculosis</i> var. <i>hominis</i>	A.T.C.C. No. 607	.007
<i>Staphylococcus aureus</i>	F.D.A. No. 209	.02
<i>Streptococcus fecalis</i>	Laboratory isolate	.08
<i>Bacillus metiens</i>	Levine	.05
<i>Serratia marcescens</i>	Laboratory isolate	.04
<i>Escherichia coli</i>	Laboratory isolate	>.3
<i>Pseudomonas aeruginosa</i>	Laboratory isolate	>.3

pathogenic fungi which were tested. Yeast forms and the tubercle bacilli are less sensitive. Several gram-positive bacteria have about the same resistance as the non-pathogenic fungi; gram-negative organisms are generally quite resistant.

*T. interdigitale* was used as the test organism for determining the static action of the compounds listed in Table IV. A physical constant for identification is given for each. The results may be compared with those reported in Table I for the straight chain fatty acids since the data were gathered under similar conditions. An examination of the results obtained with the various 6- and 11-carbon acids reveals that the point of unsaturation has little or no effect on activity. Fatty acids with multiple unsaturations (the "dienoic" acids) are about equal in activity to the singly unsaturated compounds. Branching of the carbon chain decreases the activity if one considers the total number of carbon atoms in the acid. The effect of branching is less, however, when one considers only those carbon atoms in the longest chain. The aldehyde, acetate, ethyl ester, amide and substituted amide have considerable activity but are less active than the corresponding acid. Alcohols have high activity but, due to limited solubility, the effectiveness of long chain alcohols can be demonstrated only on the more sensitive organisms.

The introduction of a phenyl group for 6 of the carbon atoms of an 11-carbon acid decreases the activity markedly regardless of the degree of unsaturation in the fatty acid chain. Substitutions in the phenyl ring with an amino or hydroxyl group further depress the activity. The presence of a cyclohexyl group on the fatty acid chain results in activity comparable to the corresponding straight chain acids. No variation which we have made in the arrangement of the carbon atoms has brought about greater activity than that displayed by the straight chain acid.

Since early times sulfur has been considered efficacious in the treatment of fungous diseases of plants and animals. As this action has been ascribed to colloidal sulfur as well as to its oxy-acids and to hydrogen sulfide, a number of related sulfur-containing compounds were included in the table. Since a sulfur atom is considered equivalent to  $-\text{CH} = \text{CH}-$ , n-heptylmercaptoacetic acid may be regarded as the isostere of an unsaturated 11-carbon fatty acid. Neither this compound nor a variety of other substituted mercaptoacetic acids showed activity of the same magnitude as the fatty acids. With the exception of 11-thiol-

TABLE IV

*Inhibition of T. interdigitale at pH 6.5 by Compounds Related to the Fatty Acids*

Compound	M.p. or b.p.	Inhibiting Concentration per cent
2-Hexenoic Acid	32°	.08
2,4-Hexadienoic Acid (Sorbie Acid)	132°	.08
2-Hendecenoic Acid	138-140°/2 mm.	.004
3-Decenoic Acid	118-120°/1.5 mm.	.007
2,12-Tridecadienoic Acid	163°/1 mm.	.002
4-Ethyloctanoic Acid	115°/2 mm.	.03
4-Ethyl-2-octenoic Acid	120°/3 mm.	.015
4-Ethyl-2-hexenoic Acid	107°/3 mm.	.05
2-Ethylhexanoic Acid	227°/760 mm.	.06
2-Ethylhexanol	182-185°/760 mm.	.04
2-Ethylhexanal	163-165°/760 mm.	.08
n-Octanol	195-198°/760 mm.	.015
Undecylenyl Alcohol	120°/5 mm.	.001
Undecylenaldehyde	94°/2 mm.	.05
Undecylenyl acetate	125-127°/7 mm.	.0075
Ethyl undecylenate	125°/8 mm.	.05
Undecylenoyl amide	85-87°	.01
Undecylenoyl thiourea	123.5-124.5°	>.3
S-undecylenoyl thiouronium chloride	120.5-121.5°	.2
N-hydroxyethylundecylenoylamide	68°	.01
5-Phenylvaleric Acid	58-59°	.08
5-Phenylpentenoic Acid	148°/1 mm.	.05
5-Phenyl-2,4-pentadienoic Acid	165-166°	.04
5-(p-Aminophenyl)valeric Acid	113-114°	>.3
5-(p-Hydroxyphenyl)valeric Acid	118-120°	>.3
5-Cyclohexylvaleric Acid	137°/1 mm.	.009
5-Cyclohexylpentenoic Acid	64-66°	.004
Ethylmercaptoacetic Acid	123-124°/15 mm.	.25
Cyclohexylmercaptoacetic Acid	140°/2 mm.	.25
n-Heptylmercaptoacetic Acid	40°	.02
11-Hydroxyethylmercaptohendecanoic Acid	66-68°	.08
11-Phenylmercaptohendecanoic Acid	81.5-82.5°	>.4
Ethane-1,2-bis (11-mercaptopohendecanoic Acid)	114-115°	>.4
11-Thiolhendecanoic Acid	48-49°	.007
11,11'-Dithiodihendecanoic Acid	84-85°	.2
10-Carboxydecylsulfonic Acid	52-53°	>.3
Thiourea	182°	.07

hendecanoic acid, none of the other thiohendecanoic acids approached undecylenic acid in activity.

### Fungicidal Results

A typical killing curve obtained with undecylenic acid is reported in Table V. Such results are reproducible although the precision is not

TABLE V

*Fungicidal Action of Undecylenic Acid in .02 M Phosphate Buffer at pH 6.5 and 37°C.*  
Approximately 100,000 *T. interdigitale* spores per ml.

Acid concentration per cent	Surviving spores/ml. after:					
	5 Min.	10 Min.	30 Min.	1 Hr.	2 Hrs.	3 Hrs.
.05				200	100	10
.075				75	0	0
.10	6000	4500	2000	17	0	0
.15	200	10	0	0		
.2	0	0	0	0		

sufficiently great for accurate computation of the time-concentration curve. It appears that within the solubility range it will approximate

TABLE VI

*Fungicidal concentrations of Fatty Acids*

Acid	Percentage concentrations killing at 2 hours		
	C Atoms	<i>A. niger</i>	<i>T. interdigitale</i>
Palmitic	16	>.2	>.2
Myristic	14	>.2	>.2
2-Tetradecenoic	14*	>.2	.01
Tridecanoic	13	—	.01
2,12-Tridecadienoic	13*	>.2	.02
Lauric	12	>.2	.015
2-Dodecanoic	12*	>.2	.02
Hendecanoic	11	.15	.05
Undecylenic	11*	.15	.06
Capric	10	.2	.13
2-Nonenoic	9*	.5	.2
Caprylic	8	.4	.45
Heptylic	7	1.3	.8
Caproic	6	5.0	2.0
2-Hexenoic	6*	5.0	3.0
Propionic	3	>25.0	25.0

\* Unsaturated acids

the conventional equation

$$\log \text{time} = m \log \text{concentration} + a$$

where  $m$  is considerably greater than unity, indicating a fairly high concentration coefficient.

From experiments involving a series of concentrations of various fatty acids and a series of time intervals the concentration of the fungicidal agent which would kill in two hours was computed. The results obtained with *A. niger* as well as *T. interdigitale* (Table VI) follow reasonably well the fungistatic results, indicating that the killing is merely a more drastic expression of a disturbance which is the same as, or very similar to, that producing stasis. The general picture is one of increased activity with increase in the length of the carbon chain to a point where the solubility becomes the limiting factor.

### SUMMARY

A number of saturated and unsaturated fatty acids and related compounds were tested for their fungistatic and fungicidal activity. The long chain saturated and unsaturated fatty acids were superior to other acids and related derivatives both in their inhibition of fungous growth and killing of fungous spores.

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# Differentiation of Rubber and Gutta Hydrocarbons in Plant Materials

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Received April 23, 1945

## INTRODUCTION

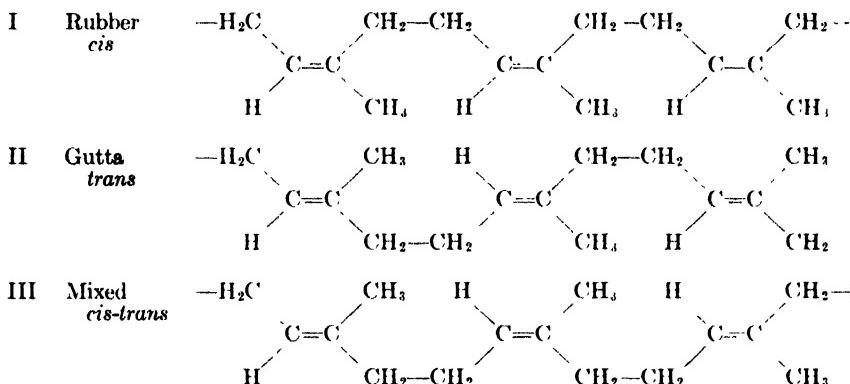
The isomeric hydrocarbons, gutta and rubber, are obtained commercially from only a few plants. They occur, however, in amounts varying from traces to major constituents in many plants and it has been an objective of this Bureau to evaluate such sources. In several instances, the isolated hydrocarbons could not be definitely identified by their elastic properties, which were intermediate between rubber and gutta. Thomas A. Edison, whose notes are on file in the Office of Rubber Plant Investigations, also experienced this difficulty. He examined some 17,000 plant samples comprising more than 1000 genera in his search for a native source of rubber. Using solvent extraction, Edison often isolated a polymerized hydrocarbon which he was unable to designate as being either rubber or gutta. Work reported here was undertaken to clarify this matter and to provide an initial approach to the biochemical problem in the manner in which such polymers are made by plants.

Rubber and gutta have the same empirical formula,  $(C_5H_8)_x$ , but differ in the spatial positions of their carbon atoms about the double bonds as shown by Formulas I and II, rubber being the *cis*- and gutta the *trans*-isomer for chain continuation (1). It was felt that the intermediate elasticity of some samples might be the result of a simple mixture of rubber and gutta or due to the presence of a "mixed" molecule in which both the *cis*- and *trans*- forms might appear (Formula III).

Chemical reactions are of little value in the identification of the isomers since the same end products are obtained when the double

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bonds, the only reactive parts of the molecules, are destroyed. Absorption of light and solubility behavior in hydrocarbon solvents are more promising methods for differentiating the compounds. Rubber and gutta absorb light only in the infra-red and far ultra-violet regions of the spectrum. In the ultra-violet, where light absorption is determined by electronic transitions, significant differences are not to be expected. In the infra-red, however, differences between rubber and gutta occur since light absorption is a result of vibrations of all the atoms in a molecule. These vibrations are to a great extent independent of the number of repeated units in a polymerized material, but are influenced by the type of isomerization within a unit.



## EXPERIMENTAL

### INFRA-RED ABSORPTION

Infra-red transmission measurements were made with a non-recording spectrophotograph located at the Palmer Physical Laboratory of Princeton University, for the use of which we are indebted to Professors L. G. Smith and Walker Bleakney. The spectrophotograph is of the rock salt prism type and utilizes a thermopile for the detection of radiation. The thermopile is connected in series with a sensitive galvanometer from the mirror of which light is reflected into a photoelectric amplifier which operates a second galvanometer. Transmitted energy is proportional to the deflection of this second galvanometer. A "globar" rod heated by alternating current is used as a source of radiation. On account of the spectral energy distribution from the source, it is necessary to increase the slit widths as the wave length increases. Such adjustments were made at selected points along the wave length scale.

Films for transmission measurements were made by placing several drops of a 2% hydrocarbon solution in a volatile solvent on a carefully polished rock salt plate ( $1.5 \times 5$  cm.). After evaporation of the solvent, the process was repeated two or

three times until a uniform film of the order of 0.02 mm. in thickness was obtained. The plate and its adhering film were placed in a holder arranged to be introduced into the light path of the spectrograph.

Transmission values,  $T$ , were obtained at intervals of 0.05 to 0.15  $\mu$  from measurements of the fraction of light transmitted by the film. These values were reduced to corresponding opacities,  $K$ , by use of the equations:

$$I_t = I_0 10^{-K}, \quad \text{or} \quad -\log \frac{I_t}{I_0} = -\log T = K$$

in which  $I_0$  is the incident energy (initial galvanometer deflection) and  $I_t$  is the transmitted energy (galvanometer deflection with the film in the light path). The opacity,  $K$ , is equal to the product of the linear absorption coefficient,  $a$ , and the thickness,  $t$ ; i.e.,  $K = at$ . Calculation of opacities is only physically significant for films of uniform thickness and absolute absorption coefficients can not be calculated unless the film thickness is known. An effort was made to obtain uniform films, and repetition of measurements on Balata gutta and Hevea rubber indicated that uniformity in thickness was adequately attained. Absorption coefficients,  $a$ , of various samples can best be compared since they are independent of thickness,  $t$ , and combine linearly instead of logarithmically as do the transmissions. Accordingly, all absorption coefficients were reduced to the same relative scale as obtained from a sample of purified Hevea rubber. This was done for gutta by proportionate displacement along the ordinates (relative opacities) until the curve, Fig. 1, was brought into general juxtaposition with that of Hevea rubber. Where measurements were made only in the region of 12.0  $\mu$ , opacities and therefore absorption coefficients were reduced to the same relative scale by adjusting them to agree with curves for rubber and gutta at 12.25  $\mu$ , that is, where the curves cross.

#### *Preparation of Hydrocarbons and Infra-Red Measurements*

Rubber obtained from *Hevea brasiliensis* latex was used as a polymerized hydrocarbon having the *cis*-configuration. Non-rubber constituents were removed by anaerobic bacterial action. The dried coagulum was dispersed in benzene and precipitated with acetone. Repeating this process four times produced a material nearly colorless in benzene solution. Balata gutta (*Mimusops balata*) was used as representative of a *trans*-compound. A sheet of balata was extracted with hot water, the hydrocarbon dispersed into benzene, centrifuged, and the hydrocarbon precipitated from the clear centrifugate with an equal volume of acetone. The precipitated gutta was washed with acetone and twice recrystallized from petroleum ether.

Spectrographic results obtained from these two hydrocarbons are shown in Fig. 1. A significant difference in absorption coefficients occurs in the region 11.8 to 12.3  $\mu$ , where, at 12.0  $\mu$ , the relative

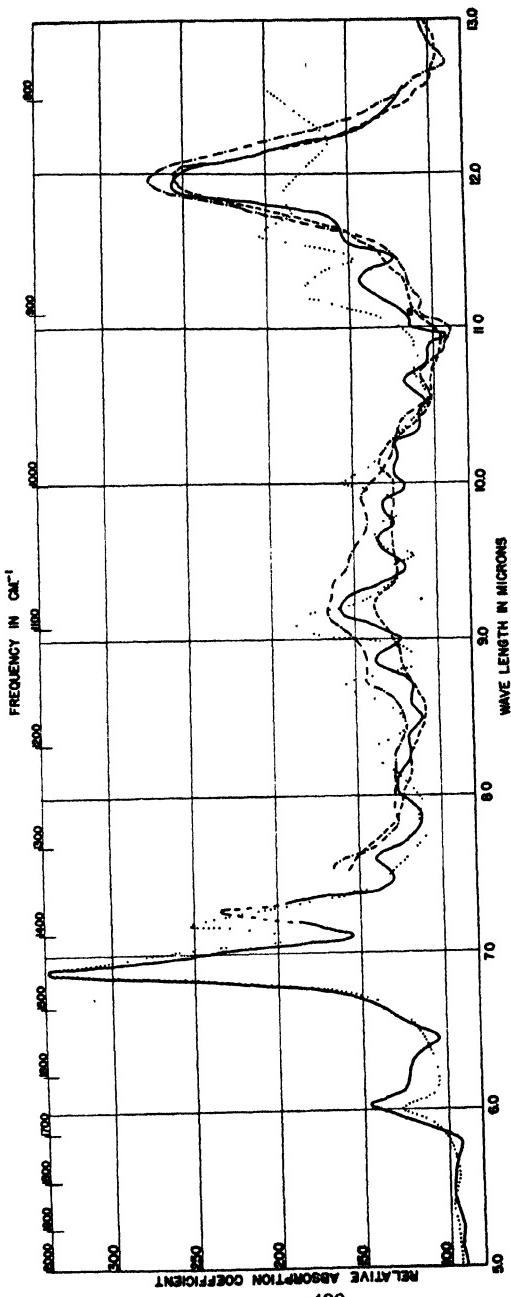


FIG. 1  
Infra-Red Absorption Spectra of Hydrocarbons from *Hevea brasiliensis* (Rubber—), *Mimusops balata* (Gutta · · · · ·),  
*Solidago leavenworthii* (Goldenrod — — —), *Jatropha* sp. (Chile - - - - -).

absorption coefficient of rubber is 42% greater than for gutta (2). These measurements and the ones for gutta are in close agreement with those of Stair and Coblenz (3) which are the only other ones previously reported. Two other samples of purified crepe Hevea rubber gave essentially the same results in the region of 11.3 to 12.5  $\mu$ , the only region measured. Therefore, a clear-cut method is offered for differentiating between rubber and gutta, and hence between *cis*- and *trans*-isomerisms in rubber hydrocarbons involving intra- or intermolecular mixtures.

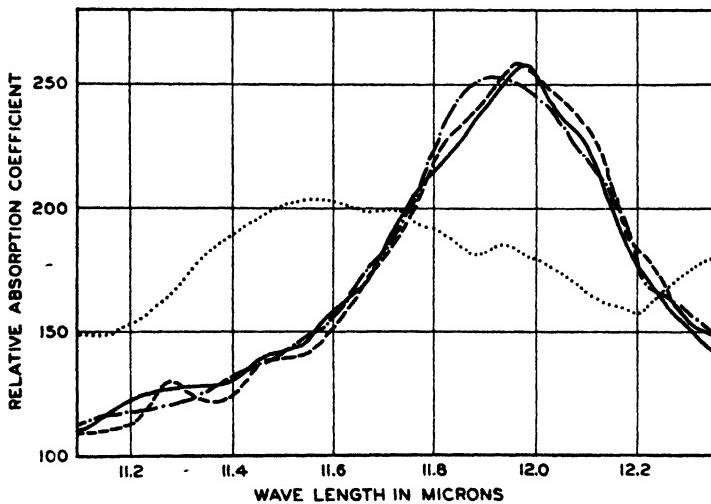


FIG. 2

Infra-Red Absorption Spectra in the Region of 12  $\mu$  of Hydrocarbons from the Milkweeds, *Asclepias erosa* (— — — —) and *Asclepias syriaca* (--- - - -) Compared with Rubber (—) and Gutta (· · · ·).

Absorption measurements in the region of 11.0 to 13.0  $\mu$  of prepared mixtures of gutta and rubber containing 10, 20, and 40% rubber, respectively, showed that less than 10% of rubber could readily be detected. One of the samples of gutta used in this work showed considerably greater absorption in the region of 11.24  $\mu$  than did the standard sample. This absorption is probably due to oxidation, which is the first step toward the more seriously degraded material studied by Stair and Coblenz (3) (their old rubber).

Two species of milkweed were examined. *Asclepias erosa* latex obtained from Arizona was evaporated to dryness and exhaustively extracted, first with acetone and then benzene. The hydrocarbon in the benzene solution was precipitated with acetone, dried, dissolved in carbon tetrachloride, and used for spectrographic analysis. *A. syriaca* was collected locally and the hydrocarbon prepared in the same manner as for *A. erosa*. Both hydrocarbons were considerably

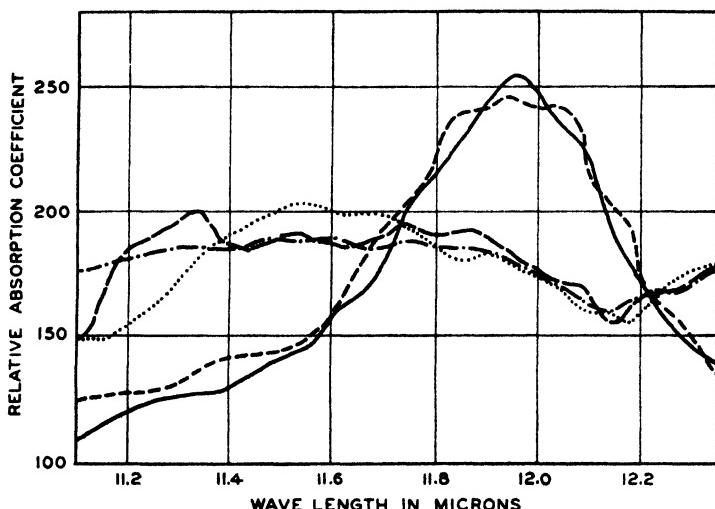


FIG. 3

Infra-Red Absorption Spectra in the Region of  $12 \mu$  of Hydrocarbons from *Eucommia ulmoides* (— · — · —), *Euonymus japonica* (— · — · · —) and *Oenothera biennis* (Evening Primrose - - - -) Compared with Rubber (—) and Gutta (· · · · ·).

less elastic than benzene-extracted Hevea rubber. Fig. 2 shows, however, that both compounds have the same absorption coefficients as rubber in the region 11.3 to  $12.5 \mu$ .

The hydrocarbon of evening primrose (*Oenothera biennis*) was extracted from dried leaves with benzene after thorough acetone extraction. It was recovered by evaporating the benzene solution to dryness. Absorption coefficients relative to Hevea rubber are shown in Fig. 3. This plant, like the milkweeds, appears to make rubber to the exclusion of gutta.

A sample of goldenrod (*Solidago leavenworthii*) hydrocarbon, obtained through the courtesy of the Southern Regional Research Laboratory of the Bureau of Agricultural and Industrial Chemistry in New Orleans, was examined. The hydrocarbon was precipitated from a 10% solution in benzene by addition of ethanol. It was twice dissolved in benzene and reprecipitated with acetone. The final product was exceedingly sticky and had only a very slight amount of elasticity. Yet, as Fig. 1 shows, the absorption coefficients are nearly the same as those found for Hevea rubber, and this compound must be considered to have the *cis*-configuration.

An opaque and rather inelastic sample of chilte rubber (*Jatropha sp.*) was dissolved in benzene, centrifuged, and the hydrocarbon thrown out of solution by addition of acetone. The sample was then swelled in petroleum ether and a very small residue (about 1%) removed by centrifuging. The petroleum ether solution was used for preparing films. Relative absorption coefficients, as shown in Fig. 1, agree closely with those of rubber, and there can be but little doubt that the hydrocarbon has the *cis*-configuration. The reduced elasticity is probably associated with the large amount of resinous substances found in the rubber.

There has been some doubt about the nature of the hydrocarbon obtained from *Eucommia ulmoides*. After extraction of a large amount of locally procured bark, first with acetone and then with benzene, the hydrocarbon regained from benzene was treated in the same manner as described for balata gutta. The final product had the appearance of an excellent grade gutta percha. In Fig. 3, the good agreement between relative absorption coefficients for *Eucommia* hydrocarbon and gutta from balata is illustrated. The agreement is close except in the region of  $11.5 \mu$  where there is a small deviation which is probably due to some oxidation. Whether this difference is reflected in the considerably higher melting point of *Eucommia* gutta ( $78^\circ\text{C}.$ ) as compared to balata ( $58^\circ\text{C}.$ ) is not known. The infra-red spectra, however, show that both of these compounds have the *trans*-type of isomerism in spite of some differences in physical properties. *Eucommia* is being exploited in Russia as a source of high-grade gutta (4).

On several different occasions, Edison examined *Euonymus japonicus* without being able to decide whether the hydrocarbon he obtained was rubber or gutta. To clarify this matter, leaves collected locally were dried and powdered, and then extracted with acetone and

benzene. The hydrocarbon, precipitated by addition of acetone to the benzene solution, was redissolved in benzene and fractionally precipitated with acetone. The final three-quarters of the material was retained for experimentation. Examination of Fig. 3 shows that the absorption coefficients of this hydrocarbon agree within limits of experimental error with those of gutta from balata and Eucommia. Other species of Euonymus are being utilized as sources of gutta in the Soviet Union (4).

#### SOLUBILITY EXPERIMENTS ON RUBBER-GUTTA MIXTURES

Previous work on the solubility of rubber and gutta in petroleum ether suggested the possibility of using this solvent to effect their quantitative separation. Sol rubber readily dissolves and gel rubber swells in petroleum ether. Gutta, on the other hand, can be recrystallized from petroleum ether.

Hevea rubber in the form of smoked para sheet was allowed to swell for 7 days in petroleum ether, after which the mixture was centrifuged and the resulting clear solution of sol rubber retained for further use. Balata gutta, purified as described previously, was dissolved in hot petroleum ether and then added in various proportions to fractions of the sol-rubber solution. The rubber-gutta solutions were then cooled to  $-8^{\circ}\text{C}$ . and examined after 30 minutes for evidence of crystallization. Gutta separated quantitatively, with greater than 98% regain, from a solution in which it was present in 1/4 the concentration of sol rubber. It was also observed to separate at 0.04 and 0.02 concentrations relative to rubber in approximately 2% rubber solutions.

Because of its opaqueness and reduced elasticity compared to Hevea rubber, chilte hydrocarbon was examined for a possible gutta fraction by solubility behavior in petroleum ether. No material separated from 1.0 and 2.0% solutions held at  $-8^{\circ}\text{C}$ . for 2 hours. Gutta which was added readily separated in  $1\frac{1}{2}$  hours at  $-8^{\circ}\text{C}$ . from 1.0% chilte rubber solutions in petroleum ether in which the concentrations of added gutta were 0.50, 0.25, 0.10, 0.05, 0.02, and 0.01 of that of the rubber hydrocarbon. It would appear from these experiments and infra-red measurements that chilte rubber could not contain more than 1.0% gutta, if any. Insolubility of gutta in cold petroleum ether, even in the presence of rubber, thus appears to offer a ready method for its preliminary identification.

The great difference in solubilities of rubber and gutta in petroleum ether is indeed noteworthy, considering their closely similar composi-

tions. In view of this, it was of further interest to examine the solubility of gutta in rubber in the absence of solvent. Films were prepared by evaporation of rubber dissolved in benzene to which various amounts of gutta had been added. Separation of gutta in the dried films was readily detected by the presence of birefringent material in the unstretched films, using a petrographic microscope. Films containing 25, 50, and 75% gutta were translucent, markedly less elastic than rubber, and highly birefringent. A film containing as little as 1% gutta was noticeably birefringent although its plastic properties were not obviously different from those of rubber.

Measurements of refractive indices and densities on films of rubber, and rubber-gutta mixtures gave further information that gutta is not mixed with chilte and goldenrod rubber. Values of these constants at 25°C. are:

	Density	Index of refraction, $n_D$	Reference
Rubber	0.906	1.5190	(5)
Gutta	0.961	1.557	(6)

The density and refractive index of a substance are related by the Lorentz-Lorenz expression:  $(n_D^2 - 1)/(n_D^2 + 2) = K_a \rho$ , where  $K_a$  is a constant characteristic of the material and is equal to the molecular refractivity divided by the molecular weight. To a close approximation,  $K_a$  of rubber and gutta are equal since their molar refractivities and molecular weights are both multiples of a C<sub>6</sub>H<sub>8</sub> unit. In the first approximation, the refractive index of a rubber-gutta film in which the gutta is immiscible will depend linearly on the composition. Thus, 1% of immiscible gutta should raise the refractive index of a film to 1.5194.

The refractive index was determined with an Abbé Zeiss refractometer used as a total reflectometer (7). Densities were measured for small portions of the films by determining the density of an acetone-water solution in which they remained suspended. The results shown below clearly support the previous evidence for the insolubility of gutta in rubber and its absence in chilte and goldenrod rubber.

Substance	Density	Observed $n_D$ at 25°C.	Calculated $n_D$
50% rubber—50% gutta		1.531	1.538
75% rubber—25% gutta		1.527	1.528
Chilte rubber	0.909	1.519	
Goldenrod rubber	0.906	1.519	

## DISCUSSION

Hydrocarbons isolated from the various plants were all definitely of the rubber or gutta types without evidence of inter- or intra-molecular mixing. Physical properties of hydrocarbons (rubber = r, gutta = g) from many other species of plants of the *Asclepiadaceae* (r), *Apocynaceae* (r, g), *Moraceae* (r), *Euphorbiaceae* (r), *Compositae* (r), *Celastraceae* (g), *Sapotaceae* (g), and other families, leaves but little doubt that rubber or gutta alone *generally* is made in the plants. A specific plant would appear to have an enzyme or enzymes necessary for the linear condensation of a hydrocarbon polymer precursor exclusively in the *cis*- or in the *trans*-configuration even though both are unstable with respect to an intra-molecular equilibrium mixture. This is, of course, the condition generally found for both geometrical and optical isomers in living systems.

The ratio ( $x$ ) of *cis*- to *trans*-forms at equilibrium is given by

$$x = \exp (-\Delta F^\circ / RT)$$

where  $\Delta F^\circ$  is the standard free energy difference between the two forms. This ratio is unknown for a rubber-gutta mixture, but it can be estimated from two independent reactions in which similar groups are involved. One of these is the isomerization of oleic to elaidic acid for which the equilibrium mixture at 170°C. in the absence of solvents contains about 20% oleic acid (8). The corresponding value of  $\Delta F^\circ$  is 1200 calories per mole. Similarly, the difference in the heats of bromination for *cis*- and *trans*-2-butene (9) in the gas phase at 30°C. is about 1000 cals. per mole and the entropy difference between the two forms is probably small. Values for  $x$  and the percentage of the *cis*-form at equilibrium for various values of  $\Delta F^\circ$  are given in the following table (temperature = 300° abs.).

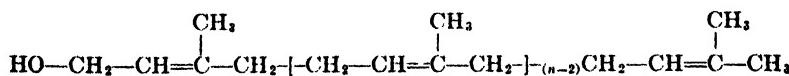
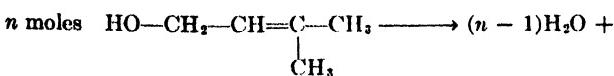
$\Delta F^\circ$ (cal./gram molecular weight)	$x = \frac{\text{conc. } cis\text{-form}}{\text{conc. } trans\text{-form}}$	% <i>cis</i> = $\left( \frac{x}{1+x} \right) 100$
0	1.000	50
600	0.368	27
1200	0.135	12
1800	0.050	4.8
2400	0.018	1.8
6000	0.000045	0.0045

A rubber-gutta mixture thus should contain 10 to 25% of the *cis*- or rubber-like configuration at equilibrium.

Two experimental methods were used in attempts to establish equilibrium between the *cis*- and *trans*-forms. The first of these made use of iodine as a possible catalyst for the isomerization of rubber to the equilibrium mixture. It failed, apparently because of loss of unsaturation due to cross linking between groups. The second method depended upon equilibrium being produced, at the time of formation, about the double bonds in commercial synthetic polyisoprene. This material might involve polymerization of isoprene elements through either end of the isoprene molecule. Cross linking between groups had also occurred in the sample examined, which swelled rather than dissolved in benzene. Films made from the small amounts that dissolved in benzene gave infra red absorptions that were generally similar to rubber and gutta, but which were modified as expected for oxidized materials.

Rubber and gutta probably utilize the same precursor and the only departure in their biosynthesis might be in the reaction which introduces or preserves the double bond of the polymerizing unit. Dehydrogenation after polymerization seems unlikely since the chance would exist for failure of the reaction to be completely carried out, resulting in reduced unsaturation of the final product. Rubber and gutta have the same empirical formula,  $(C_6H_8)_n$ , and appear to contain one double bond to each unit within less than one part in a thousand (10). If the isomerization is introduced before polymerization, at least two distinctly different enzyme systems would be required, one to differentiate the formation of the *cis*- or *trans*-precursor and the other to join the resulting *cis*- or *trans* form into linear polymers.

Polymerization of a precursor to form rubber or gutta is rather similar to the polymerization of glucose by  $\alpha$  or  $\beta$  glycosidic linkages to form starch or cellulose. While both starch and cellulose are generally present in a plant, a mixed condensation of glucose by  $\alpha$  and  $\beta$  linkages has not been observed. A probable situation in the case of rubber and gutta is that isomerism is introduced into the hydrocarbon molecule at the time of polymerization of the five carbon precursor. These conditions can be fulfilled by a reaction of the following type:



## SUMMARY

Hydrocarbons of the rubber (r) or gutta (g) types isolated from *Hevea brasiliensis* (r), *Mimusops balata* (g), *Asclepias erosa* (milkweed (r)), *A. syriaca* (milkweed (r)), *Oenothera biennis* (Evening primrose (r)), *Solidago leavenworthii* (goldenrod (r)), *Eucommia ulmoides* (g), *Euonymus japonicus* (g), and *Jatropha* sp. (chilte (r)) were positively identified by their light absorptions near 12  $\mu$ . Mixed *cis*- and *trans*-isomers were absent in all cases even though the plants examined were selected as giving hydrocarbon polymers of questionable types.

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# Determination of the Cystine and Methionine Content of Plant and Animal Material by a Differential Oxidation Procedure \*

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Received April 28, 1945

## INTRODUCTION

The sulfur-containing amino acids, cystine and methionine, are of importance in animal nutrition and metabolism since sulfur can only be utilized by the animal when obtained as cystine (1, 2) or methionine (3). There has been much interest in the cystine content of foods and feeds because cystine has long been recognized as an important constituent of animal proteins, particularly the keratins. Recent work has established the fact that methionine can be converted to cystine by the animal (4) so that at present a knowledge of both the cystine and methionine content of feeds and foods is of value in a study of the protein nutrition of man and domestic animals.

Recent work on the vegetable protein concentrates has shown that soybean oil meal and peas are deficient in available methionine for the growing chick when fed as the sole source of protein (5, 6). Grau and Almquist (7) have set the cystine plus methionine requirements of growing chicks at 1.0–1.1 per cent of the diet; at least 0.5–0.6 per cent methionine must be present in the diet.

Considerable data are available in the literature on the cystine and methionine content of purified proteins. Such data are of only limited value in determining the cystine and methionine content of practical diets since the proteins studied make up only a portion of the total feed or food proteins. Attempts have been made to adapt the methods used in analyzing pure proteins for the determination of the cystine and methionine of complex materials. The results obtained are of

\* Published as Scientific Paper No. 648, College of Agriculture and Agricultural Experiment Stations, State College of Washington, Pullman.

questionable value owing to considerable destruction of the amino acids during acid hydrolysis in the presence of carbohydrates (8).

Blumenthal and Clarke (9) observed that fuming nitric acid oxidized cystine to sulfate, but did not oxidize methionine to sulfate. Lugg (10) applied this principle to the determination of the cystine and methionine in purified proteins. Callan and Toennies (11) found that an alkaline permanganate solution oxidized cystine sulfur but not methionine sulfur to sulfate.

Evans and St. John (12) observed that about 96 per cent of the sulfur of cystine and 7 per cent of the sulfur of methionine were oxidized to sulfate by a short nitric and perchloric acid digestion. This same procedure oxidized less of the sulfur in herring fish meal, soybean oil meal, and ground wheat to sulfate than did oxidation with alkaline permanganate (13). It appeared possible, because of the above results, that concentrated nitric acid might be used to oxidize cystine sulfur to sulfate in the differential oxidation procedure replacing the fuming nitric acid that was used by Blumethal and Clarke (9) and Lugg (10) or the alkaline permanganate solution of Callan and Toennies (11).

### EXPERIMENTAL

The differential oxidation procedure that was finally adopted as a result of the data presented later in this paper (Table I) was a modification of the method of Blumenthal and Clarke (9). Three sulfur fractions were determined on each protein concentrate studied. Total sulfur was determined by the Parr bomb method (14) or the nitric and perchloric acid digestion method (12). Inorganic sulfur was determined by precipitation and weighing as barium sulfate of the sulfate sulfur liberated during hydrolysis with dilute hydrochloric acid (15). The inorganic plus cystine (sulfur oxidized to sulfate by nitric acid) was determined as follows:

Weigh a 2.00 g. sample of protein concentrate into a 500 ml. Kjeldahl flask, add 35 ml. of conc. nitric acid, and heat on the steam bath 24 hours. Wash into a 250 ml. beaker containing about 0.5 g. of potassium nitrate and evaporate to dryness on the steam bath. Add 10 ml. of conc. hydrochloric acid and again evaporate to dryness. Dissolve in distilled water containing 1.0 ml. of conc. hydrochloric acid. Filter, precipitate and weigh the sulfate as barium sulfate.

Cystine and methionine are calculated by the following equations:

$$\text{Cystine} = 3.747 \times (\text{sulfate S after nitric acid oxidation} - \text{inorganic S}).$$

$$\text{Methionine} = 4.651 \times (\text{total S} - \text{sulfate S after nitric acid oxidation}).$$

The factors 3.747 for cystine and 4.651 for methionine were calculated from the theoretical sulfur content of these amino acids. For example, methionine contains 21.5 per cent sulfur. One hundred divided by 21.5 equals 4.651, the factor used.

The equations are based on the assumptions that (1) cystine and methionine are the only organic sulfur compounds present, and (2) cystine sulfur is oxidized to sulfate while methionine sulfur is not.

The validity of the method of oxidizing cystine but not methionine sulfur to sulfate was determined in the following experiment.

TABLE I

*Oxidation of Cystine and Methionine to Sulfate by Concentrated Nitric Acid*

Sample	Cystine or methio- nine added	Weight of sulfur added*	Weight of sulfur obtained as sulfate†	Added sulfur recovered	Sulfur recovered
Cystine and dextrose	0.050	0.0133	0.0123	0.0123	92.5
Methionine and dextrose	0.050	0.0106	0.0000	0.0000	0.0
Soybean oil meal	—	—	0.0055	—	—
Soybean oil meal and cystine	0.050	0.0133	0.0187	0.0132	99.3
Soybean oil meal and methionine	0.050	0.0106	0.0056	0.0001	0.9
Fish meal	—	—	0.0090	—	—
Fish meal and cystine	0.050	0.0133	0.0221	0.0131	98.5
Fish meal and methionine	0.050	0.0106	0.0093	0.0003	2.8

\* Calculated from the sulfur content of the methionine and cystine samples used as determined by Parr bomb analysis for total sulfur.

† Includes sulfur from cystine or methionine and soybean oil meal or fish meal. The dextrose contained no sulfur.

Five-hundredth g. samples of cystine or methionine were added to 2.0 g. samples of dextrose, soybean oil meal and herring fish meal which were oxidized with nitric acid as described above. The results are presented in Table I. For these samples 92.5, 99.3 and 98.5 per cent, respectively, of the added cystine sulfur was recovered as sulfate, while 0.0, 0.9 and 2.8 per cent of the added methionine sulfur was recovered as sulfate. It appears that considerable organic matter was necessary for cystine sulfur to be completely oxidized to sulfate, since nitric acid oxidized only 62.4 per cent of cystine sulfur to sulfate when no dextrose was added. When 0.5 g. of dextrose was added, 81.7 per cent of the cystine sulfur was oxidized to sulfate. Ninety-two and five tenths per cent of the cystine sulfur was oxidized to sulfate by nitric acid when 2.0 g. of dextrose were added with the cystine.

The methionine contents of the soybean oil meals and the cottonseed meals were also determined by the colorimetric procedure of McCarthy and Sullivan (16). The values by this method agreed with those by the differential oxidation procedure when corrections were made for losses of methionine during acid hydrolysis and inhibition of color by interfering substances. Soybean oil meal protein contained an average of 1.4 per cent methionine by the differential oxidation procedure compared to 1.5 per cent by the colorimetric procedure. Cottonseed meal contained 1.8 compared to 1.9 per cent.

The values for the cystine and methionine content of a number of different plant and animal products were determined by the differential oxidation procedure as outlined in this paper and are presented in Table II. These values are converted to a protein basis in Table III.

TABLE II  
*Cystine and Methionine Content of Some Plant and Animal Materials*

	No. Samples	Cystine		Methionine	
		Range		Range	
		per cent	per cent	per cent	per cent
Casein	2	0.43	0.45	0.44	2.44
Whey	1	—	—	0.32	—
Herring fish meal	2	1.35	1.40	1.38	2.21
Pilchard fish meal	3	1.08	1.33	1.17	1.69
Dogfish meal W.P.	4	1.04	1.18	1.12	1.45
Dogfish meal D.P.	1	—	—	1.37	—
Meat scraps	2	0.62	0.95	0.79	0.71
Soybean oil meal	12	0.87	1.03	0.93	0.59
Cottonseed meal	2	0.78	1.18	0.98	0.71
Alaska peas	2	0.24	0.28	0.26	0.18
First and best peas	2	0.24	0.32	0.28	0.21
Alah peas	1	—	—	0.34	—
Corn	1	—	—	0.23	—
Oats	1	—	—	0.32	—
Wheat	2	0.26	0.29	0.28	0.23
Barley	1	—	—	0.15	—
Millrun	1	—	—	0.32	—
Alfalfa	1	—	—	0.35	—

## DISCUSSION

A comparison of the data presented in Table III with data previously published on the cystine and methionine content of similar plant and animal proteins is of interest. Casein contained 0.5 per cent cystine. Folin and Looney (17) reported 0.3 per cent, Teruuchi and Okabe (18) reported 0.3 per cent, Kassel and Brand (19) reported 0.4 per cent, Hess and Sullivan (20) reported 0.3 per cent, and Beach *et al.* (21) reported 0.3 per cent cystine in casein. Casein contained 2.9 per cent methionine. Kassel and Brand (19) reported 3.1 per cent, and Beach *et al.* (21) reported 3.1 per cent methionine in casein.

Less information is available regarding the cystine and methionine content of the total protein in more complex plant and animal ma-

terials. Block and Bolling (22) and Grau and Almquist (23) have presented the most extensive data. (See Table III.) Gubler and Greaves (24) reported 0.4 per cent cystine in wheat compared with the 0.3 per cent reported here. Hamilton and Nakamura (25) reported 0.2-0.6 per cent cystine in soybeans compared with the 0.9 per cent

TABLE III

*Cystine and Methionine Content of Some Common Plant and Animal Proteins\**

	No Sam- ples	Cystine		Values from the Litera- ture (22)	Methionine		Values from the Literature	
		Range	Aver- age		Range	Aver- age	(22)	(23)
		per cent	per cent		per cent	per cent	per cent	per cent
Casein	2	0.5	0.5	0.5	2.8	3.0	2.9	3.6
Whey	1	—	—	2.6	—	—	3.2	2.1
Herring fish meal	2	1.8	2.0	1.9	3.0	3.1	3.1	3.1
Pilchard fish meal	3	1.7	1.9	1.8	2.8	3.0	2.9	—
Dogfish meal, wet process	4	1.5	1.7	1.6	2.1	2.3	2.2	—
Dogfish meal, dry process	1	—	—	1.7	—	—	1.4	—
Meat scrapes	2	1.1	1.7	1.4	1.0	1.2	1.5	1.4
Soybean oil meal	12	1.8	2.1	2.0	1.0	1.3	1.6	1.4
Cottonseed meal	2	2.1	2.6	2.4	—	1.7	1.9	1.8
Alaska peas	2	1.1	1.2	1.2	—	0.8	1.6	1.2
First and best peas	2	0.9	1.3	1.1	—	0.8	1.1	1.0
Alash peas	1	—	—	1.4	—	—	0.9	—
Corn	1	—	—	2.3	—	—	—	3.1
Oats	1	—	—	3.0	—	—	—	2.2
Wheat	2	2.4	2.7	2.6	—	2.1	2.8	2.5
Barley	1	—	—	1.7	—	—	—	3.7
Millrun	1	—	—	2.2	—	—	3.2	—
Alfalfa	1	—	—	1.8	—	—	2.0	2.3

\* Protein = N × 6.25.

in soybean oil meals or 2.0 per cent cystine and 1.4 per cent methionine in the soybean protein reported in this paper. Tomiyama (26) reported 1.1 per cent cystine and 2.0 per cent methionine in the soybean protein. Grau and Almquist (23) reported 2.2 per cent methionine, and Block and Bolling (22) reported 1 per cent cystine and 2.0 per cent methionine in soybean protein.

Other comparisons between the results reported in this paper and those of Grau and Almquist (23) and Block and Bolling (22) show that in general the cystine reported here is higher and the methionine lower than reported by them. (See Table III.) Block and Bolling (22) determined cystine by two colorimetric procedures on acid hydrolysates of the fat free materials and the values obtained are probably low due to losses during acid hydrolysis. Part of their methionine results were calculated from the non-cystine organic sulfur and would thus

be high when the cystine was low. Grau and Almquist (23) determined methionine by the method of McCarthy and Sullivan (16) on acid hydrolysates prepared by partially hydrolyzing the material with 6 N HCl or pepsin. Damodaran and Krishnaswamy (27) by the use of one colorimetric method obtained high cystine values on peptic digests of proteins when compared with another colorimetric method or with values obtained on acid hydrolysates of the protein by either method. Work in this laboratory indicates the presence of something in enzyme digests of soybean oil meal which interferes with the McCarthy and Sullivan (16) reaction for methionine. There was not enough cystine present in the soybean oil meal to cause the fading observed by White and Koch (28). Another cause of difference might well be differences in the composition of the proteins studied. That such differences occur is shown by the different cystine and methionine content of the protein of different samples of peas (Table III).

From the data here presented the adequacy of some common diets in cystine and methionine can be calculated. For example, Grau and Almquist (7) give the requirements of growing chicks as 0.5 per cent methionine and 1.0 per cent methionine plus cystine in the diet. A chick starting mash containing 20 per cent protein, 11 per cent protein coming from soybean oil meal and 9 per cent from cereal grains would contain about 2.4 per cent methionine and 2.1 per cent cystine in the protein, using the values of 2.1 per cent cystine and 3.3 per cent methionine for the protein of the cereal grain mixtures. This is equal to about 0.48 per cent methionine and 0.90 per cent methionine plus cystine in the diet. This diet would therefore be on the borderline and probably slightly deficient in methionine and methionine plus cystine. If the 11 per cent of supplementary protein were furnished by herring fish meal, the mash protein would contain about 3.2 per cent methionine and 1.9 per cent cystine or 0.64 per cent methionine and 1.02 per cent methionine plus cystine in the diet. This contains sufficient methionine and total sulfur amino acids if all are available. The value of other protein concentrates can be calculated in the same manner.

#### SUMMARY

A method is described for the determination of the cystine and methionine content of complex plant and animal products based on the differential oxidation procedure of Blumenthal and Clarke. The

method used consisted of the determination of three sulfur fractions: total sulfur, inorganic sulfur, and the sulfur oxidized to sulfate by heating with concentrated nitric acid. It was shown that cystine sulfur is oxidized to sulfate but methionine is not by heating with nitric acid in the presence of considerable organic matter.

On the protein basis, the cereals, fish meals and casein were the best sources of methionine studied, and the cereals, whey, fish meals, soybean oil meal and cottonseed meal the best sources of cystine. Proteins of peas and the meat scrap were the poorest sources of both amino acids.

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# Re-investigation of the Relative Provitamin A Potencies of Cryptoxyanthin and $\beta$ -Carotene

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Received June 5, 1945

## INTRODUCTION

Recently our group reported on some experiments concerning the influence of stereochemical changes on the provitamin A activity of carotenes; in all, 598 rats were used (3). It seemed desirable to re-investigate the influence of the introduction of a hydroxyl group into an all-*trans* carotene molecule by applying the same methods and a similar stock of animals as before.

For this purpose a hydroxy- $\beta$ -carotene, the well known cryptoxyanthin,  $C_{40}H_{56}\cdot OH$ , was the obvious test material. Kuhn and Grundmann (7) have reported that this pigment "caused in vitamin-A-free rats in doses of 5 to 10  $\mu g$ . per day about the same growth as  $\beta$ -carotene in 2.5  $\mu g$ . doses." In their important paper, it was evidently not intended to establish a more exact quantitative relationship between the two respective potencies; neither was the number and the distribution of the animals indicated. However, the procedure itself is available from an earlier article by Brockmann and Tecklenburg (1). Eight years later Fraps and Kemmerer (4) have stated that the effect of  $\beta$ -carotene and cryptoxyanthin (IU per  $\mu g$ . of pigment) corresponds to a ratio of 1.1:0.6; no details concerning the levels administered or the number of animals were given. Finally, Schormüller (8) seems to have published some data which are, however, not available to us.

In the present paper we wish to report on a more extensive evaluation of the ratio mentioned. As summarized in Table I, two independent series of assays were carried out, in February–March and April–May, 1945. In all 135 rats from our own stock colony were used.

TABLE I

*Summary Table of Bioassays on Rats Receiving Cryptoxanthin or  $\beta$ -Carotene in Cottonseed Oil or the Oil Alone (Negative Controls)*

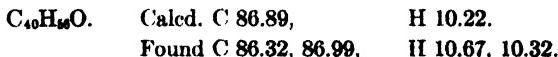
(The average results on males and females are weighted equally. Where animals died during the course of the experiments, the number of animals still alive which are included in the average, is given in parentheses. The average age at the start was 22-23 days, and the average duration of the depletion period varied between 22 and 26 days.)

Supple- ment	Dose per day	Number of rats		Ave- rage start- ing weight	Assay period							Po- tency ( $\beta$ - caro- tene = 100)	
					Average increase in body weight up to following days								
		Male	Fe- male		5	10	15	20	25	Total 28	Aver- age final weight	Aver- age age of death	
<i>Series I.</i>	$\mu\text{g.}$			g.	g.	g.	g.	g.	g.	g.	g.	days	per cent
$\beta$ -Caro- tene	0.5	7	7	96.0	1.5	11.0	19.8	25.5	31.6	33.2	128.7	—	—
	1.0	5	6	90.0	6.8	22.0	32.0	42.0	49.5	54.6	144.6	—	—
Crypto- xanthin	0.6	4	6	94.2	-1.2	8.1	12.6	17.4	21.2	22.8	117.0	—	—
	1.2	7	6	88.8	2.0	14.2	21.5	28.8	37.6	41.4	130.2	—	(60)* 54
Negative controls	0.0	5	7	93.2	-1.9	-1.7	-5.7	-7.8 (10)	-6.9 (8)	-1.2 (5)	96.6 (5)	22.1 (7)	—
<i>Series II.</i>	$\beta$ -Caro- tene	0.5	8	7	99.6	1.5	10.6	17.6	21.0	25.0	26.6	126.2	—
	1.0	7	8	101.4	1.1	14.5	24.0	33.2	39.8	42.3	143.7	—	—
Crypto- xanthin	0.6	8	7	97.8	-0.8	6.5	12.4	16.8	20.6	20.6	118.4	—	—
	1.2	8	7	96.6	4.5	13.1	20.8	26.8	31.2	34.4	131.0	—	(84)* 59
Negative controls	0.0	7	8	99.8	-4.9 (14)	-1.3 (14)	-1.0 (14)	-6.1 (13)	-10.7 (9)	-14.9 (7)	87.7 (7)	21.8 (8)	—

\* The values given in parentheses should be considered as unreliable since we had to extrapolate the  $\beta$ -carotene curves (Fig. 1) considerably in order to be able to calculate these values.

## EXPERIMENTAL

The technique of the bio-assays and connected procedures was described earlier (3). The pure cryptoxanthin crystals (m.p. 170°, cor.) originated from fruits and petals of *Physalis Alkekengi* and *Ph. Franchetti*, which were collected in Southern Hungary.



The sample proved to be chromatographically homogeneous. It separated easily from either  $\beta$ -carotene, zeaxanthin, or physaliene on the calcium hydroxide column.

## RESULTS

The absolute level of the effective cryptoxanthin doses (like that of  $\beta$ -carotene) lies considerably lower than observed by Kuhn and Grundmann (7). Indeed, in the present tests practically a maximum response was obtained on a dose as small as one-eighth of their maximum dose or one-fourth of the minimum dose reported by these investigators. Since the augmentatory effect of  $\alpha$ -tocopherol was only recognized later (6), Kuhn and Grundmann had not added this compound to the supplements. Our animals received 0.5 mg. of  $\alpha$ -tocopherol daily which may be the main cause for the divergency just mentioned. (The synergism between  $\beta$ -carotene and vitamin E,

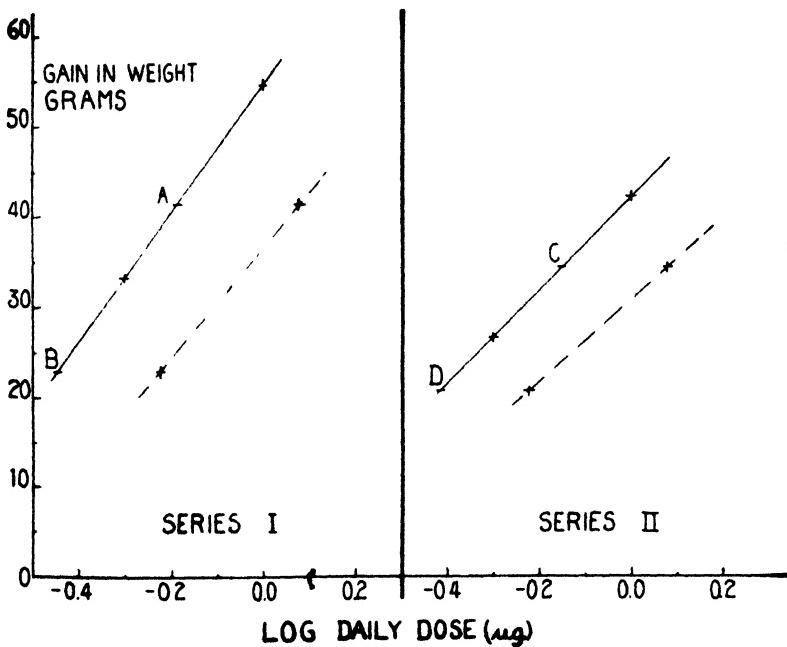


FIG. 1

Relationship of Gain in Weight to Log. of Daily Dosage of  
 $\beta$ -Carotene and Cryptoxanthin

Solid lines represent the curves obtained for  $\beta$ -carotene and dashed lines those for cryptoxanthin. Points A and B are the projections on the  $\beta$ -carotene curve of weight increase of rats receiving daily 1.2 or 0.6  $\mu$ g. of cryptoxanthin, respectively, in Series I. Points C and D refer to Series II.

as mixed tocopherols, has been questioned recently by Gridgeman (5) although no details of his experiments have as yet been reported.)

Calculating according to Coward (2) (cf. Fig. 1), we found the ratio of the provitamin A effects,  $\beta$ -carotene: cryptoxanthin, to be 100:54 and 100:59 in the two independent series of experiments. Thus the average potency of cryptoxanthin amounted to 57 per cent to that of  $\beta$ -carotene, in good accordance with the result reported by Fraps and Kemmerer (4).

We conclude that the following alterations of the all-*trans*- $\beta$ -carotene molecule result in approximately an equal decrease of the provitamin A potency: migration of a double bond out of conjugation (yielding  $\alpha$ -carotene); one *trans*  $\rightarrow$  *cis* rearrangement near the end of the conjugated system; or the introduction of one hydroxyl group into all-*trans*- $\beta$ -carotene in the usual position which is *meta* to a quaternary carbon atom. Only slightly stronger is the weakening effect of two *trans*  $\rightarrow$  *cis* rotations, if one of them takes place in the middle section of the chromophore (3).

### SUMMARY

The relative provitamin A potencies of  $\beta$ -carotene,  $C_{40}H_{56}$ , and cryptoxanthin,  $C_{40}H_{56}\cdot OH$ , were re-investigated using 135 rats. This ratio was found to be 100:54 and 100:59 in two independent series of bioassays.

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# Desaminases for Ribosenucleic and Desoxyribosenucleic Acids\*

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Received June 7, 1945

Solutions of either sodium yeast (ribosenucleic) nucleate or sodium thymus (desoxyribosenucleic) nucleate, when incubated aerobically at neutral pH with tissue extracts, do not split off appreciable amounts of inorganic phosphate (1) (2), nor do they yield organic products from the nucleates capable of passing through a cellophane membrane (2). Under the conditions noted, considerable physical changes in the nucleates occur, *i.e.*, a decrease in viscosity of the thymus nucleate and in the acid-precipitability of the yeast nucleate, without degrading the respective nucleates to particles sufficiently small to pass readily through the membrane (less than about 40 Å in diameter). In connection with recent studies involving the effect of nucleates on dehydrogenase systems (3) we had occasion to confirm these findings, but it was noted at the time that ammonia was progressively split off from the nucleates when the latter were incubated with extracts of certain tissues. The possibility that there exist in certain animal tissues desaminases for the nucleates when the latter are still in the form of fairly large molecules was therefore investigated.

Mixtures of equal volumes of fresh rat spleen extract in distilled water (equivalent to 160 mg. tissue per cc.) with a 0.5 per cent aqueous solution of yeast or of thymus nucleate were prepared and incubated at 37°C. for varying lengths of time. There occurred a progressive formation of ammonia in the digests which was estimated by aeration of the alkalinized digests into acid traps with subsequent nesslerization (4) (5). Controls of the extracts alone were simultaneously made and were taken into account. The sodium nucleates were highly purified products used earlier in physico-chemical studies (*cf.* 2) and in aqueous solution were almost completely non-dialyzable through cellophane. The results, including studies of rat kidney extracts, are illustrated in Fig. 1.

\* Preliminary Report.

Each of the nucleates, whether of the yeast or the thymus variety, possesses three amino groups per tetranucleotide unit, belonging respectively to the adenine, the guanine and the cytosine moieties. If all three moieties are desaminated, it would be expected that 5 mg. of the nucleate would yield close to  $150 \times 10^{-3}$  mg. of ammonia

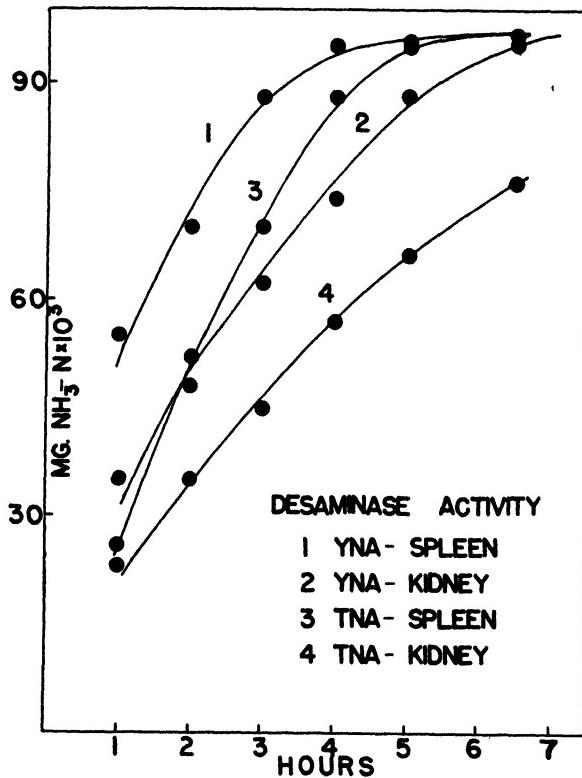


FIG. 1

Nucleodesaminase activity of aqueous extracts of rat spleen and of kidney (equivalent to 160 mg. tissue per cc.) as a function of time of incubation at 37°C. Mixtures consist of 1 cc. extract plus 1 cc. of either yeast or thymus sodium nucleate (0.5 per cent in water). Ordinate refers to mg. ammonia N  $\times 10^3$ , abscissa refers to hours of incubation. Curve 1 describes yeast nucleate plus spleen extract; curve 2, yeast nucleate plus kidney extract; curve 3, thymus nucleate plus spleen extract; and curve 4, thymus nucleate plus kidney extract. Value of 100 on ordinate scale corresponds to two atoms of ammonia N per tetranucleotide unit. Initial and final pH practically identical, *i.e.*, 6.8–7.0.

nitrogen. The results in Fig. 1 show however that a maximum of nearly  $100 \times 10^{-3}$  mg. of ammonia nitrogen is attained and, therefore, only two out of the three available amino groups are hydrolyzed. Which one of the three moieties is resistant to desamination has not been determined at the present time but we are inclined to believe that it may be the cytidine (6) (*cf.* 7).

Similar studies under identical conditions of the desamination capacities of other tissues for the nucleates revealed that the order of activity was spleen > kidney > nodes > brain > liver. Muscle extracts were negative. For the same tissue in different species, the order was rat > mouse > guinea pig > rabbit. In every case the rate of desamination for the yeast was greater than for the thymus nucleate (*cf.* Fig. 1). Studies of the desamination capacities of the above tissues for adenylic acid (yeast), adenine, guanine and guanosine, revealed the universal presence of a guanosinase, the universal absence of an adenase, and a distribution of adenylic acid desaminase and of guanase entirely different from that of the nucleate desaminases. Thus, although liver extract rapidly desaminates adenylic acid and guanine, it has only a very weak action on either of the nucleates. The capacity of the extracts to desaminate any of the substrates is destroyed by brief heating at 100°C.<sup>1</sup>

No added buffers were employed in the enzyme studies since tissue extracts were used, and, as will be seen subsequently, it was necessary to study the effects of individual ions on the activity of the respective desaminases. A study was nevertheless made of the pH of the digests of rat spleen and of kidney with each of the nucleates at the beginning and at the end of a four hour period of incubation at 37°C. (*cf.* legend to Fig. 1). The initial pH of each of the mixtures was 6.8–7.0, and the final pH was practically unchanged from these values.

The desaminases in aqueous spleen extract for the two kinds of nucleates may be clearly distinguished by the following (using mixtures of rat tissue and sodium nucleates described above): (a) the enzymatic desamination of yeast nucleate is partially, that of thymus nu-

<sup>1</sup> We made the observation in this connection that whereas aqueous tissue extracts ordinarily yield almost immediately a coagulum of protein on heating at 100°C., such extracts mixed previously in the amount of 160 mg. tissue plus 5 mg. sodium thymus nucleate per cc. yield no coagulum whatever on heating at 100°C. for several hours. Yeast nucleate caused a similar but much less pronounced effect. This phenomenon will be the subject of a future communication.

cleate entirely, inhibited by 0.01 *M* fluoride, (b) progressive dilution of the extract with distilled water results in a more rapid falling off of the capacity to desaminate thymus than yeast nucleate, and (c) when the aqueous tissue extracts, *as well as the nucleate solutions*, were each thoroughly dialyzed against distilled water and then mixed and incubated for four hours, the capacity to desaminate yeast nucleate was unchanged whereas the capacity to desaminate thymus nucleate disappeared. The latter capacity could be completely restored to the dialyzed extract by the addition of the halides (except fluoride), sulfates, nitrates, and acetates of any one of the following ions at 0.01–0.001 *M* concentration: Li<sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, Rb<sup>+</sup>, Cs<sup>+</sup>, Ca<sup>++</sup>, Mg<sup>++</sup>, Sr<sup>++</sup>, Ba<sup>++</sup>, Mn<sup>++</sup>, Ni<sup>++</sup>, Co<sup>++</sup>, Fe<sup>++</sup>. Equally effective were the halides of such organic bases as arginine and guanidine. Beryllium salts, as well as salts of silver, mercury and trivalent metals, were not only ineffective in the dialyzed but toxic in the fresh extract. The nature of the anion of the effective restorative salts appears to be unimportant except when such anion is fluoride, bicarbonate, or cyanide, in which case the salt is ineffective. The surprising effectiveness of the salts of the organic bases led to a study of simple amino acids and peptides which may be considered to be inner salts, but neutral compounds such as glycine, glycylglycine and others at 0.01 *M*, failed to restore the desamination capacity of the dialyzed rat spleen extract for thymus nucleate. Equimolar mixtures of an otherwise activating salt, such as sodium chloride, with a beryllium salt were ineffective in restoring thymus nucleodesaminase activity to the dialyzed tissue extract. The effectiveness of the guanidine salts suggests a link with protein metabolism.

The sharp separation through dialysis in the capacity of the tissues to desaminate the two kinds of nucleate is noteworthy. Equally striking is the ability of so many diverse ions to restore the activity of the dialyzed tissue desaminase for thymus nucleate—a property which at the moment may be regarded as exceptional.<sup>2</sup> Although the thymus as well as the yeast nucleate was employed as the sodium salt, the concentration of this metal was always below the effective

<sup>2</sup> It is possible that the recognition of many similar phenomena of ion activation of various kinds of enzymic systems may have been obscured by the nearly universal practice of adding electrolyte buffers, sometimes unnecessarily, to incubation mixtures.

activating range (minimum of 0.001 *M* for 5 mg. nucleate and equivalent of 160 mg. tissue).

The desamination of the nucleates *in situ* would lead to molecules in which such bases as xanthine and hypoxanthine would be present in place of the parent aminated purines, and thus purine metabolism might occur under natural circumstances at the nucleic acid level. It is proposed that the enzymes responsible for desaminating the nucleic acids *in situ* be separately designated ribonucleodesaminase and desoxyribonucleodesaminase. Complete details together with studies of tumors will be published elsewhere (8).

*Added in proof:* Lineweaver and Ballou have recently made interesting observations on the activation of dialyzed solutions of alfalfa pectinesterase by a variety of alkali or alkaline earth salts (Lineweaver, H., and Ballou, G. A., *Arch. Biochem.* **6**, 373, (1945)).

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## Rhodanese

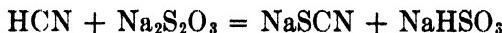
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Received June 6, 1945

### INTRODUCTION

In 1933 Lang (1) discovered an enzyme in animal tissues which causes the formation of rhodanate from thiosulfate, or colloidal sulfur, and hydrocyanic acid. Lang found the reaction to be:



The enzyme was present in highest concentration in kidneys and liver but absent from muscle and blood. Reversibility of the reaction could not be demonstrated. Lang named the enzyme "rhodanese," since it synthesizes rhodanate. Starting from cow liver, he was able to purify rhodanese about twenty-fold. His procedure was to adsorb upon kaolin and to elute with disodium phosphate. He found that the reaction catalyzed by rhodanese follows the Schütz rule, especially if the concentration of cyanide employed is not greater than 0.005 M. His measuring rhodanese activity depended on a colorimetric determination of the rhodanate after adding ferric nitrate in a "Stupho" photometer.

We have verified many of Lang's findings. However, we have preferred to devise a method of our own for measurement of rhodanese activity. Lang employed phosphate buffer at pH 8.3 in his substrate, but since phosphate is hardly a buffer at this pH we have used phosphate of pH 7.4. This is reasonably near the pH optimum for rhodanese. We have found that the reaction catalyzed by rhodanese does not follow the Schütz rule, contrary to Lang's statement, no matter what dilution of cyanide is present in the substrate. Finally, we have been able to purify beef liver rhodanese about 100-fold.

## EXPERIMENTAL

### *Purification of Rhodanese*

Liver is disintegrated in a "Powermaster" blender, using 100 ml. of ice water for every 100 g. of liver. To every 100 ml. of the brew 24 ml. of chloroform and 24 ml. of 95% alcohol are added and the mixture shaken violently for about 20 seconds (2). The preparation is then centrifuged in 500 ml. bottles for about 1 hour in a cold room. This procedure removes a large amount of protein. The supernatant solution is dialyzed in the cold against several changes of 0.001 *M* neutral phosphate buffer for one or two days to remove all chloroform and alcohol. To every 100 ml. of the dialyzed rhodanese solution 2 ml. of 9.6% neutral phosphate solution and 110 ml. of neutral, saturated ammonium sulfate is added and centrifuged in the cold after mixing. The clear supernatant liquid is mixed with 100 ml. of neutral, saturated ammonium sulfate and allowed to stand in the cold in 500 ml. centrifuge bottles overnight. The next day as much as possible of the clear supernatant liquid is sucked off and discarded. The remainder is centrifuged at high speed. After discarding the supernatant, the precipitate is dissolved in dilute neutral phosphate buffer. This rhodanese solution will be found to have been purified about 100-fold.

### *Determination of Rhodanese Activity*

One ml. of the rhodanese solution is pipetted into a large test tube, graduated at the 50 ml. level, and placed in a bath at 20°C. Four ml. of cyanide-thiosulfate-phosphate solution at 20°C. is then added from a small graduated cylinder and mixed. After a 5-minute interval the reaction is stopped by adding 5 ml. of ferric nitrate in dilute nitric acid and mixing. The test is diluted to 50 ml., stoppered and mixed. A blank is prepared by diluting 1 ml. of enzyme, 4 ml. of cyanide-thiosulfate-phosphate and 5 ml. of ferric nitrate to 50 ml. and mixing. Both test and blank are now allowed to stand for 15 minutes so that the violet color which ferric iron gives with thiosulfate may have time to fade completely. Using a blue filter, the Klett-Summerson or Fisher colorimeter is now set at the zero point with the blank solution, after which the unknown solution is read. This reading is used to obtain the ml. of 0.01 *N* rhodanate from a standard graph. This graph is prepared by making solutions containing from 1 to 16 ml. of 0.001 *M* KSCN, 4 ml. of cyanide-thiosulfate-phosphate and 5 ml. of ferric nitrate up to 50 ml., and reading them in the colorimeter, using the above mentioned blank to obtain the zero point. Here also, it is necessary to wait 15 minutes for the violet color to fade. The standard curve is a straight line.

Our rhodanese units are expressed in ml. of 0.01 *N* rhodanate formed by the enzyme in 5 minutes at 20°C.

### Rhodanese Activity and the Schütz Rule

As shown in Fig. 1., rhodanese activity is proportional to the quantity of rhodanate formed, provided the latter does not amount to more than 15 ml. of 0.001 *N*, and provided the cyanide concentration is not too low. When the cyanide concentration is as low as 0.004 *M*, however, the quantity of rhodanate formed falls off relatively with increasing quantities of enzyme, as is shown in the graph.

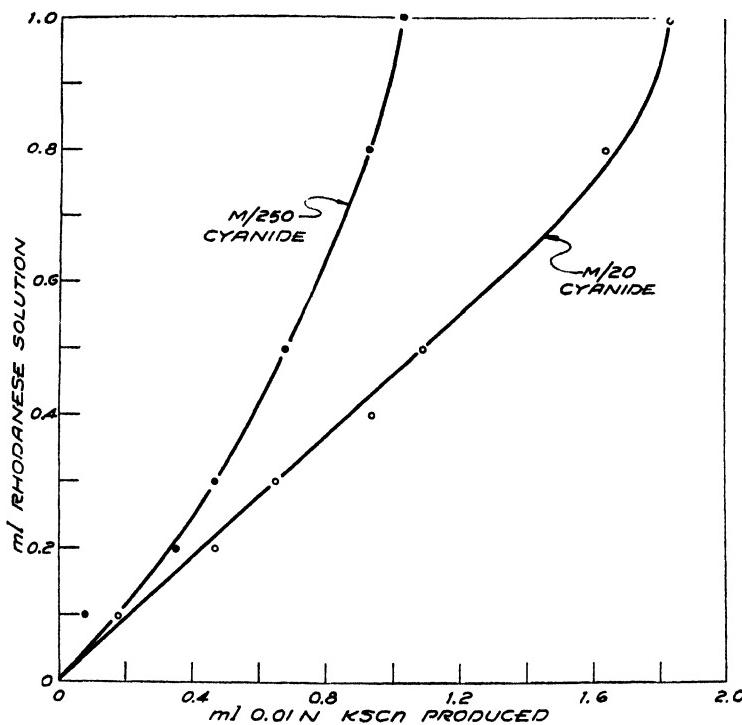


FIG. 1

Table I shows that the Schütz rule does not apply to the reaction catalyzed by rhodanese, since the ml. of rhodanate formed divided by the square root of the ml. of rhodanese solution used do not give a constant value.

### Preparation of Reagents

The substrate is prepared by dissolving 6.5 g. of potassium cyanide (Kahlbaum, "for analysis") in about 1 liter of water. A few drops of phenol red are added and

TABLE I

Ml. of rhodanese	Ml. KSCN formed	
	$\sqrt{\text{Ml. rhodanese}}$	0.05 M cyanide    0.004 M cyanide
0.1	0.57	0.26
0.2	1.05	0.80
0.3	1.19	0.85
0.4	1.49	—
0.5	1.54	0.96
0.8	1.83	1.04
1.0	1.84	1.30

enough *N* sulfuric acid (about 100 ml.) to give a neutral reaction, followed by 170 ml. of 0.5 *M*  $\text{Na}_2\text{HPO}_4$  and 30 ml. of 0.5 *M*  $\text{KHPO}_4$  and 24.8 gm. of sodium thiosulfate. When everything has dissolved, the solution is diluted to 2 liters and mixed. This reagent must be kept in the ice chest, as the constituents soon react at room temperature to produce rhodanate. The ferric nitrate solution is prepared by dissolving 50 g. of  $\text{Fe}(\text{NO}_3)_3 \cdot 7 \text{ H}_2\text{O}$  in water, adding 100 ml. of colorless, concentrated nitric acid, diluting to 1 liter and mixing.

We wish to express our thanks to the Rockefeller Foundation for financial support.

### SUMMARY

A procedure is described by means of which beef liver rhodanese can be concentrated about 100-fold.

A method is presented for determination of rhodanese activity.

Contrary to the findings of Lang, our data show that the reaction catalyzed by rhodanese does not follow the Schütz rule, regardless of the concentration of cyanide employed.

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# Sensitization of Pneumococcus Type 1 Towards Penicillin by Specific Anti-pneumococcus Serum

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Received June 11, 1945

## INTRODUCTION

In a previous paper from this laboratory, Buck and Schnitzer (1) described the synergistic effect of a combination of type specific anti-pneumococcus serum and penicillin in experimental infections with Type 1 and Type 2 pneumococci. In these experiments, a subtherapeutic dose of the immune serum injected 4 hours before the infection was combined with a single small ineffective dose of penicillin sodium salt (20 to 30 units per 20 g. mouse = 1/2 to 1/3 the minimal active dose) given subcutaneously shortly after the intra-abdominal infection with 1000 m.l.d. of the pneumococci.

It was found later on that with a strain of Type 1 pneumococcus which was rendered relatively penicillin-resistant, a striking synergism was still obtained if the same doses of anti-serum were combined with the same or higher doses of penicillin. Single injections of 30 to 100 units penicillin, as used in these experiments, represented less than 1/80 to 1/200 of the active dose. A detailed description of these investigations, carried out by M. Buck, will be published in the near future.

These observations appeared to indicate that the synergistic activity of anti-pneumococcus serum + penicillin might be interpreted as a sensitization of the pneumococci by the antibodies towards the antibacterial action of penicillin. The sensitization was apparently sufficiently effective to overcome even a certain degree of experimentally induced penicillin resistance.

In order to study the mechanism of this sensitization, an attempt was made to produce the sensitization of the pneumococci *in vitro* and to determine the susceptibility of these organisms towards penicillin *in vivo*.

## MATERIALS

The experiments were carried out with white mice weighing 16-20 g.

The strain of Type 1 pneumococcus No. 6301 and the anti-pneumococcus serum (Lederle MC 8/2/43, unpreserved) were the same as described in the previous paper (1). The penicillin sodium salt used in these experiments was taken from the residues of commercial penicillin (100,000 units per ampoule) after it had been routinely assayed in this laboratory.

Normal goat serum, not inactivated, served as comparison with the immune serum.

## EXPERIMENTAL

### *Sensitization of the pneumococci in vitro.*

An overnight serum broth of Pn. No. 6301 was plated on a set of blood agar plates. After 18 to 20 hours' incubation, the heavy growth of pneumococci was washed off with 2 cc. plain broth per plate. Four cc. each of this suspension were filled in centrifuge tubes, treated with 4 cc. anti-pneumococcus serum 1-50 dilution in saline or 1-50 diluted normal goat serum, respectively, and left standing at room temperature for one hour. After that, the tubes were centrifuged, the deposits broken up by stirring with sterile glass rods and washed one to three times with 10 cc. plain broth. The supernatants were decanted and the sediments suspended in 5 cc. plain broth. This material was used for infection of mice. Virulence titrations of the suspensions after treatment with normal or immune serum showed that no loss of virulence occurred during the procedure. Intra-abdominal infection with 0.5 cc. of a  $10^{-9}$  dilution killed mice consistently within 48 hours.

In agglutination tests carried out with the absorbed serum as well as with the final washings, no free agglutinins could be detected.

### *Penicillin treatment in vivo.*

Mice were infected intra-abdominally with 0.5 cc. of a  $10^{-7}$  dilution (= 90-150 cells/cc.) of the suspensions mentioned above. One group of mice was infected with the sensitized pneumococci, another one with the normal ones. The mice in each

TABLE I

### *Activity of Penicillin on Sensitized and Normal Pneumococci in Mice*

Infection: 0.5 cc. of a  $10^{-7}$  dilution\* of a suspension of washed organisms after treatment in vitro with immune serum and normal serum, respectively.

Pneumococcus 6301 treated with serum	Penicillin 30 units/20 g.	Number of mice	Number of survivors	Percentage survivors
Normal	1 x	32	3	9.4
	2 x	12	10	83
	0	16	0	0
Immune	1 x	32	24	75
	2 x	12	11	92
	0	16	0	0

\* In 1 experiment, 0.5 cc. of a  $10^{-6}$  dilution was given.

group were subcutaneously treated with 30 units penicillin/20 g. approximately 5 minutes after the infection. Some of the animals received a second treatment after 3 hours. Untreated mice of each group served as controls.

The results are given in Table I, in which the data of 4 experiments with identical outcome are presented.

### DISCUSSION

After contact with normal serum, the sensitivity of the pneumococci was normal; it is in accordance with our previous experience that a treatment with 2 times 30 units protected the majority of mice. A single treatment with 30 units was always practically inactive.

If, however, the strain had been in contact with specific antibodies *in vitro*, an infection of mice with the washed organisms proved to be more sensitive to treatment with penicillin. A single subcutaneous injection of 30 units protected 75% of the treated animals.

Lower doses of this antiserum (*e.g.*, a dilution 1-100) did not confer higher sensitivity to pneumococci. It was, however, possible to demonstrate the increase of sensitivity to penicillin in very heavy infections. In one experiment carried out with pneumococci which had been in contact with 1-50 immune serum, mice were infected with 0.5 cc. of a  $10^{-5}$  dilution of the washed organisms, corresponding to 800 cells. A single treatment with 30 units penicillin protected 5 out of 10 animals.

From these experiments, it appears justified to assume that a comparatively short contact of pneumococci with diluted immune serum sensitized these organisms towards the subsequent action of penicillin *in vivo*. The main sources of error to be encountered in experiments of this type; namely, loss of virulence and interference of free antibodies, perhaps cannot be excluded completely. But the plate counts as well as the virulence titrations indicated that the infection with sensitized pneumococci was not appreciably lighter than that with the normal ones. There was, moreover, no evidence that any excess of free antibodies over those adsorbed by the pneumococci was present in the highly diluted infective material. The mechanism of the sensitization is, of course, not considered to be specific towards the anti-pneumococcal activity of penicillin. There is no doubt that the well known combination effect of anti-pneumococcus serum with other drugs, such as quinine derivatives and sulfonamides, is due to

the same mechanism, which consists in the opsonic (Wright) or bacteriotropic (Neufeld and Rimpau) sensitization of pneumococci towards phagocytosis. The synergistic effect of anti-pneumococcus serum and penicillin is therefore most likely a synergism of phagocytosis and penicillin in infections with pneumococci sensitized by antibodies.

Analogous experiments with the penicillin-resistant strain have been unsuccessful so far, due to the fact that this strain underwent a considerable loss of virulence after contact with antiserum *in vitro*.

#### SUMMARY

Type 1 pneumococci, sensitized by contact with diluted specific antiserum *in vitro*, washed, and injected into mice, possessed a higher sensitivity towards the antibacterial activity of penicillin *in vivo*. The same strain of pneumococci treated with diluted normal serum showed normal sensitivity to penicillin *in vivo*.

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# Viscosimetric Determination of Thymonucleodepolymerase \*

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Received June 15, 1945

## INTRODUCTION

A method for evaluating thymonucleodepolymerase activity, based on measurements of viscosity, has been described by Greenstein (1). The difficulty in utilizing viscosity for the quantitative determination of enzymic activity lies in the fact that many non-enzymic substances influence the viscosity of thymonucleic acid (4). Greenstein (1) determined the drop in viscosity caused by an active enzyme during 30 minutes of incubation at 30°C. and subtracted from it the drop in viscosity of a control sample in which the active enzyme was replaced by a solution of horse serum albumin containing an amount of total N equal to that of the examined enzyme solution. Analysis of data, obtained by Greenstein (2) according to his previously described procedure (1), indicated that the values for thymonucleodepolymerase activity calculated for extracts of the same tissue containing 0.41 and 1.23 mg. of total N per cc. varied considerably.

It occurred to us that better results might possibly be obtained if the initial drop in viscosity caused by mixing thymonucleic acid with an inactive protein were entirely disregarded and the reaction measured from this point on. The purpose of this paper is to describe the use of a viscosimetric method in which this initial drop is ignored. In this method a further assumption is made that at any given time of incubation the relative viscosity of the reacting mixture is proportional to the concentration of the unattacked thymonucleic acid. This assumption

\* Research paper No. 564, Journal Series, University of Arkansas. Aided by a grant from the John and Mary R. Markle Foundation.

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is obviously untrue, but it is believed that under the experimental conditions used the error introduced by such an assumption is comparatively small.

The relative viscosity of the investigated mixture could not be ascribed entirely to the thymonucleic acid. Even after the enzymic reaction has been completed the viscosity of the reacting mixture would be somewhat higher than the viscosity of water due to the presence of the products of the reaction and the components introduced with the extract of the enzyme. By disregarding this value a constant error was introduced into the calculations. This error, however, was small because the influence of all non-specific components accounted for only a small fraction of the viscosity of thymonucleic acid.

The second objection to the assumption of viscosity being proportional to the concentration of thymonucleic acid in the reacting mixture is furnished by the recent findings of Cohen (3). He showed that during the digestion of thymonucleohistone by chymotrypsin, trypsin and ribonuclease the viscosity of the mixture increased due to the liberation of the free thymonucleic acid. The error due to the possible breakdown of a complex formed by the protein and nucleic acid would equally endanger the procedure of Greenstein as well as ours. This error would be highest with those extracts containing large amounts of protein and large amounts of proteolytic enzymes. Fortunately, the concentration of thymonucleic acid during the experiment is in considerable excess over the concentration of protein and, therefore, only a small part of thymonucleic acid could exist as a less viscous protein complex. The purer the solution of thymonucleodepolymerase the smaller the error due to the proteolytic breakdown of such a complex would be.

The value of the method described below is, therefore, limited to uniform and comparatively pure systems. The method has little value for determining thymonucleodepolymerase in extracts of different organs, because the introduction of large amounts of different proteins and proteolytic enzymes creates conditions impossible to control.

## EXPERIMENTAL

Thymonucleic acid was prepared according to the method of Hammarsten (4). The preparation exhibited a negative biuret reaction. Five cc. of a 0.5% solution of thymonucleic acid in 0.2 M borate buffer pH 7.0 were placed in an Ostwald viscosimeter immersed in a water bath at 37°C. The enzyme extract to be investigated was diluted with the same buffer to the appropriate strength and was warmed to the same tem-

perature. Three cc. of the diluted extract were pipetted into the viscosimeter, thoroughly mixed and the viscosity at zero time was taken. During the following 30 minutes viscosity readings were made at 5-minute intervals.

The velocity constant of a monomolecular reaction was calculated from the formula:  $K = (1/t) \log (\eta_0/\eta_t)$ ; where  $\eta_0$  = relative viscosity at 0 time,  $\eta_t$  = relative viscosity at time  $t$ .

The unit of thymonucleodepolymerase was defined as an amount of enzyme which under the previously specified conditions would give the value  $K = 1.0 \times 10^{-3}$ . The potency of the enzyme was expressed as a number of units per mg. of protein, which was determined according to the method of Robinson and Hogden (5). It could also be expressed as "thymonucleodepolymerase capability" =  $K/(g. \text{ of protein})$ ; the last term has been used in analogy to the "catalase capability" coined by Sumner (6) to replace von Euler's (7) Saccharasefähigkeit. In our case potency is numerically equal to capability.

### DISCUSSION

The record of a typical experiment is presented in Table I. It shows the normal variation of the value  $K$  when calculated for 6 subsequent periods of 5 minutes each. An average value of these 6 readings was

TABLE I  
*Record of a Typical Experiment*

	Time of incubation min.	Time of flow through viscosimeter sec.	Relative viscosity	$\frac{\eta_0}{\eta_t}$	$\log \frac{\eta_0}{\eta_t}$	$K$
Water		34	1.00	—	—	—
Thymonucleic acid	0	124	3.66			
+ extract of beef pancreas	5	120	3.53	1.03	0.0128	0.00256
	10	118	3.47	1.05	0.0212	0.00212
	15	113	3.32	1.10	0.0414	0.00276
	20	107	3.15	1.16	0.0645	0.00322
	25	105	3.08	1.19	0.0755	0.00302
	30	100	2.98	1.22	0.0864	0.00288
Average Units						0.00276
						2.76

considered to represent the activity. The variation in the value of  $K$  (Table I) did not exceed the expected experimental error and, therefore, justified the practical usefulness of the previously discussed assumptions.

The applicability of the method to the quantitative determinations of thymonucleodepolymerase in crude extracts of pancreas is shown in

Table II. In these experiments different quantities of the same extract were analyzed. An error of 25% can easily be caused by making only one determination. The averages of several determinations, however, indicate a straight proportionality between the amount of enzyme used and the value of  $K$ .

TABLE II  
*Relation Between the Value of the Velocity Constant and the Amount of Enzyme Used*

Enzyme preparation	Amount of enzyme cc.	$K \times 10^3$	$K \times 10^3$ calculated on the basis of the first value	Found Calculated $\times 100$
Hog pancreas extracted with 3 volumes of borate buffer, centrifuged and diluted to contain 1.67 mg. protein per cc.	0.5	3.96	3.96	100
	0.75	4.48	5.94	75
	1.0	6.65	7.92	94
	1.0	8.15		
	1.25	10.22	9.99	101
	1.25	9.75		
	1.50	11.69	11.88	98
Beef pancreas extracted with buffer, extract precipitated with 70% acetone and dissolved in buffer; contains 6.7 mg. protein per cc.	2.0	4.71		
	2.0	4.01	4.05	100
	2.0	3.42		
	3.0	6.88		
	3.0	7.26	6.51	6.07
	3.0	5.39		107

Greenstein and Jenrette (8, 9) and Greenstein (2) investigated the depolymerase activity of various biological materials at pH 7.0 only. It seemed of interest to determine the depolymerase activity over a wider pH range. Fig. 1 represents the results obtained in such experiments with crude extracts of hog pancreas. Fig. 2 shows the control experiments in which three-times recrystallized egg albumin was used. It is evident that the relative viscosity of the mixture of thymonucleic acid plus depolymerase dropped very slowly at either pH 5 or 6. At pH 8 during the first 5 minutes the velocity of the reaction seemed to be equal to that at pH 7 but slowed down considerably thereafter. The shape of the curves at pH 7 corresponded roughly to that of a monomolecular reaction. In the control experiments (Fig. 2) no definite drop

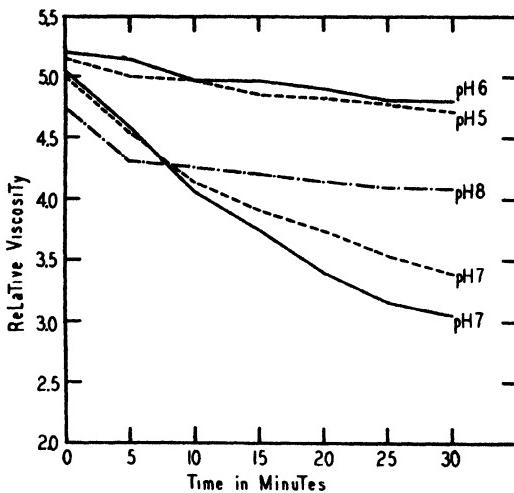


FIG. 1

Time-Activity Curves of Crude Extracts of Thymonucleodepolymerase from Hog Pancreas at Different pH Values Using Viscosity as Criterion of Enzymic Activity

In each experiment enzyme containing 1.67 mg. protein was diluted to 3 cc. with appropriate buffer and mixed with 5 cc. of 0.5% thymonucleic acid dissolved in the same buffer.

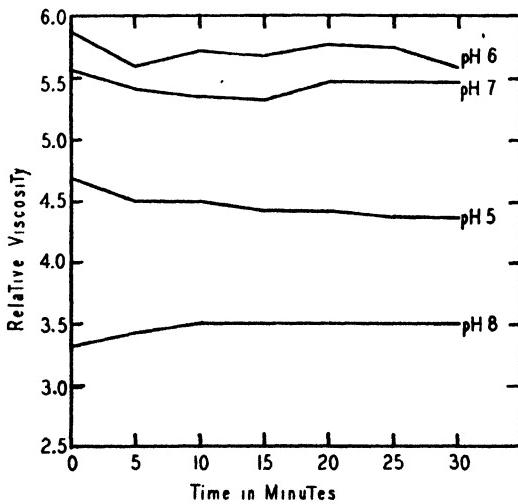


FIG. 2

Control Experiments Under Conditions Specified in Fig. 1 Except that 8.8 mg. of Three-Times Recrystallized Egg Albumin Was Used Instead of the Enzyme Solution

in relative viscosity was observed at any pH studied, and the small variations were within the range of the experimental error.

The results of the experiments shown in Fig. 1 are summarized in Fig. 3. The average  $K$  values were taken to represent the activity at a given pH. The existence of the optimum of about pH 7 was, therefore, established. The second curve shown in Fig. 3 represents the values

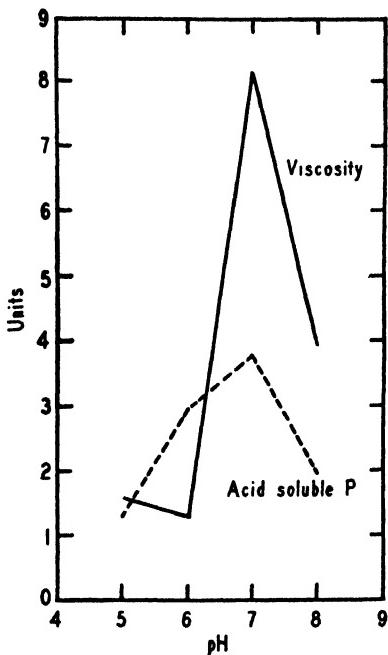


FIG. 3

Rate of Depolymerization of Thymonucleic Acid as a Function of pH

obtained by determining the liberated acid-soluble phosphorus by a method analogous to that of Kunitz (10) for the determination of ribonuclease.

The finding of Greenstein and Jenrette (8) and Avery, MacLeod, and McCarty (11) concerning the thermolability of depolymerase was confirmed. Table III shows the results of the experiments run on a slightly purified extract of hog pancreas containing 11.9 units activity and 0.5 mg. of protein per 0.1 cc. Enzyme solution was diluted to 3 cc. with 0.2 M borate buffer; the substrate was 5 cc. of thymonucleic acid (5

mg. per cc.). Five minutes exposure to 60°C. destroyed the activity completely.

TABLE III  
*Effect of Elevated Temperatures on Thymonucleodepolymerase*

Temperature	Units recovered		Per cent activity recovered	
	Exposure time 1 min.	5 min.	Exposure time 1 min.	5 min.
45°	12.7	12.6	107	106
50°	4.6	6.9	39	58
55°	4.4	6.0	36	50
60°	2.4	0.0	20	00

In the course of the experiments to be described later, it was noticed that the addition of Mg<sup>++</sup> ions strongly accelerated the activity of thymonucleodepolymerase. Table IV shows the influence of Mg<sup>++</sup> on

TABLE IV  
*Effect of Magnesium Sulfate on Thymonucleodepolymerase*

Molarity of added magnesium sulfate in reacting mixture	$K \times 10^3$
0.0	6.62
0.00125	8.19
0.00250	10.65
0.00625	13.67
0.0125	15.94
0.025	18.53
0.125	18.41

the value of  $K$ . Each sample consisted of enzyme solution containing 0.089 mg. protein of somewhat purified extract of hog pancreas, varying amounts of magnesium sulfate solution and 5 cc. of thymonucleic acid; total volume 8 cc., pH 7. The maximal effect was approached after addition of 2 cc. of 0.1 M MgSO<sub>4</sub> to the reacting mixture (final concentration of Mg<sup>++</sup> 0.025 M). A control experiment in which magnesium sulfate alone was added to the solution of thymonucleic acid did not give any measurable value of  $K$ .

The action of magnesium on thymonucleodepolymerase might have been either direct or indirect through stimulation of phosphatase and the removal of the products formed in the course of the first reaction. To determine which of these possibilities occurred, the experiment summarized in Table V was performed. Each tube was made 0.025 M in MgSO<sub>4</sub>. Other constituents are shown in the Table.

The results of this experiment definitely exclude the indirect action. The preparation of thymonucleodepolymerase used in the experiment was found to be free from phosphatase (tube II). Furthermore, the addition of phosphatase<sup>1</sup> to the mixture of thymonucleic acid and

TABLE V  
*Comparison of Thymonucleodepolymerase and Phosphatase*

	I	II	III		IV	
Enzyme	Phosphatase	Thymonucleode-polymerase	Thymonucleode-polymerase		Thymonucleode-polymerase and phosphatase	Thymonucleic acid
substrate	$\beta$ -Glycerophosphate	$\beta$ -Glycerophosphate	Thymonucleic acid	7.0	Thymonucleic acid	7.0
pH	9.0	9.0				
Time	Free P	Free P	Acid soluble P	Free P	Acid soluble P	Free P
min.						
0	273	none	78	none	165	73
30	2574	none	858	none	884	598
60	2730	none	830	none	962	910

thymonucleodepolymerase (tube IV) did not increase the liberation of acid-soluble phosphorus to any considerable degree (tube III).

On the basis of this finding the previous definition of the unit was extended to include the optimal concentration of  $Mg^{++}$  as 0.025 M.

#### SUMMARY

A method is described for the quantitative determination of thymonucleodepolymerase. It is based on measurements of the change in viscosity of the reacting mixture. The reaction is roughly of the first order and the value of the velocity constant,  $K$ , is used for the determination of enzymic activity. Fairly good proportionality is found between the value of  $K$  and the amount of enzyme used. The optimal pH of thymonucleodepolymerase is close to 7. The enzyme is activated by magnesium ions, optimal concentration being 0.025 M.

<sup>1</sup> We are greatly indebted to Dr. G. Schmidt for a generous gift of highly purified phosphatase. The finding of Schmidt and Thannhauser (12) that their phosphatase does not attack the undigested thymonucleic acid has been confirmed.

A proposed unit of thymonucleodepolymerase is defined as an amount of enzyme, which will give the value of the velocity constant  $K = (1/t)$   $\log (\eta_0/\eta_t) = 1 \times 10^{-3}$  when incubated with 25 mg. of thymonucleic acid (prepared according to Hammarsten) at 37°C., at pH 7 in 0.2 M borate buffer, made 0.025 M with respect to MgSO<sub>4</sub>; total volume of the reacting mixture 8 cc.; time of incubation 30 minutes; readings at .5 minute intervals.

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# **Electrophoretic Investigation of Peanut Proteins**

## **I. Peanut Meal Extract, Arachin and Conarachin**

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Received June 22, 1945

### **INTRODUCTION**

The first extensive investigation of peanut proteins (*Arachis hypogaea*) was conducted by Johns and Jones (1) who concluded that the total protein consisted of two globulins and a very small amount of a heat coagulable albumin. The globulins were separated by ammonium sulfate fractionation of a sodium chloride extract of peanut meal to yield a fraction, named arachin, estimated to comprise approximately 75% of the total protein and a second fraction, named conarachin, comprising the remaining protein (1, 2). Evidence that arachin and conarachin represented different proteins was based on the sharpness with which ammonium sulfate fractionation of sodium chloride extracts of peanut meal occurred and on significant differences in the optical rotations and the sulfur, basic nitrogen and lysine contents of the two fractions (1, 3). More recent work has shown that arachin and conarachin also differ markedly with respect to their methionine, cystine, threonine, tryptophane and tyrosine contents (4, 5, 6) and with respect to their ability to support growth of the white rat (7). Furthermore, Tayeau (8) has reported that the isoelectric point of arachin, as determined by the minimum solubility method, is approximately pH 5.1 to 5.2 while that for conarachin is in the range pH 3.9 to 4.0.

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Because it represents approximately two-thirds of the total protein of the peanut and can be isolated quite simply in substantial quantities, arachin has been used by many investigators as a representative protein of this oilseed. However, none of the more recently developed techniques for the characterization of proteins has been applied to either arachin or conarachin to determine their homogeneity. The results, reported in this paper, of an electrophoretic investigation of the peanut proteins and of arachin and conarachin, indicate that the peanut contains at least three, and probably four, protein components and that arachin and conarachin each consist of mixtures of at least two of these components.

### EXPERIMENTAL

*Preparation of peanut meal.* The peanut meal used in these experiments was prepared in April, 1943, by the following procedure. White skinned peanuts of the 1940 crop<sup>3</sup> were shelled mechanically and the shell fragments carefully removed. The unblanched meats were mechanically cracked and flaked and the flakes extracted with petroleum ether (boiling range, 30° to 60°C.) in a pilot-plant batch extractor at room temperature. The solvent was removed by aeration and the resulting air-dried meal ground in a hammer mill. Two preparations of solvent-extracted meal (Nos. 26F and 35G) were used in the experiments reported in this paper. Upon analysis, preparations 26F and 35G were found to have the following composition: moisture, 10.56 and 9.40%; ash, 5.14 and 5.14%; total nitrogen, 9.08 and 8.84%; non-protein nitrogen (that soluble in molar trichloroacetic acid at 2°C.), 0.59 and 0.46%; protein nitrogen, 8.49 and 8.38%, protein ( $N \times 5.5$ ),<sup>4</sup> 46.7 and 46.1%.

*Electrophoretic analysis of peanut meal extract:* One gram of peanut meal (No. 26F) was extracted successively in the cold (1° to 3°C.) with five portions of ammonia buffer (0.2 M NH<sub>3</sub> and 0.1 M HCl, pH 9.26 at 25°, 0.1 ionic strength) by alternately stirring and centrifuging, and the combined extracts were diluted with buffer to 50 ml. An alkaline buffer was used in order to obtain maximum extraction of the nitrogen in peanut meal (10) and a sufficiently high protein concentration (0.8 percent) for accurate electrophoretic analysis. The combined extracts contained an average of 98% of the total nitrogen present in the meal,<sup>5</sup> and consequently, ap-

<sup>3</sup> The white skinned peanuts used represent a composite sample of a single hybrid variety (Virginia Runner, male  $\times$  Improved White Spanish, female) developed and grown at the Georgia Coastal Plains Experiment Station, Tifton, Georgia (9, 10). These peanuts have been shown to be identical, with the exception of the seed coat color, with the more common red skinned varieties (11).

<sup>4</sup> As pointed out by Jones and Horn (2) the factor for converting total nitrogen to protein in the case of the peanut should be approximately 5.5 instead of the conventional 6.25.

<sup>5</sup> The total meal nitrogen in the extract equals Kjeldahl nitrogen minus ammonia nitrogen (due to the buffer) as determined by direct distillation in the presence of magnesium oxide. Aqueous peanut meal extracts contain only a negligible trace of ammonia nitrogen.

proximately 98% of the protein of the meal. Analytical values for the nitrogen contained in replicate extracts varied from 96.5 to 99.5% of the total meal nitrogen.

The extract was dialyzed in a cellophane membrane against 0.5 liters of ammonia buffer in the cold for 2 to 4 hours. The dialysis was continued in 1.8 liters of fresh buffer for an additional 14 to 16 hours and the dialyzed solution was then centrifuged at high speed for 15 minutes (relative centrifugal force of  $3800 \times$  gravity) to remove a minute amount of precipitate which formed during dialysis. The clear dialyzed solution contained approximately 0.9% protein or approximately 98% of the protein of the meal. Electrophoretic analysis of the dialyzed solution was carried out immediately as previously described (12).

A typical pattern for the ammonia buffer extract of peanut meal is shown in Fig. 1. Three well defined boundaries are apparent. The calculated mobilities of these boundaries, which have been designated A, B and C, are  $-6.3$ ,  $-5.1$  and  $-3.7 \times 10^{-5}$ , respectively. Estimation of the areas under the peaks by the method of Tiselius and

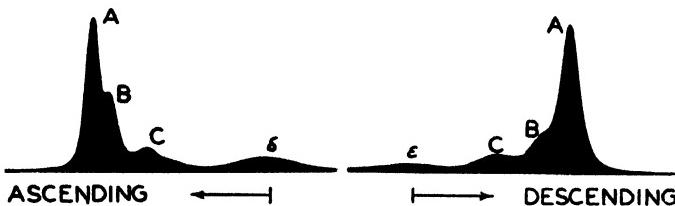


FIG. 1

Electrophoretic Pattern of an Ammonia Buffer Extract of Peanut Meal After Electrophoresis for 15,600 Seconds in Ammonia Buffer at pH 9.26

Kabat (13) indicates that component A comprises approximately 76%, B, 11% and C, 13% of the protein present in the ammonia buffer extract.

Examination of the ascending boundaries in Figs. 1 and 3, however, reveals that the peak designated as C in the descending boundaries of Fig. 1 is resolved, under certain conditions, into two boundaries. For this reason in the discussion to follow, the major protein components of the peanut will be referred to separately as component A (faster moving major component) and component B (slower moving major component), while the components responsible for the composite peak will be referred to as the "minor components."

From these results it may be concluded tentatively that a peanut meal extract, prepared in the manner indicated, contains two major protein components, A and B, which are present in the approximate

ratio 7:1 and which, together, constitute approximately 87% of the protein present in the extract. The remaining 13% of the protein probably consists of components of which two, present in approximately equal amounts, have been recognized by their mobility.

The conditions used for the extraction of the protein from the meal were selected so that opportunity for denaturation of the protein would be minimized and the amount of protein extracted would approach 100% of that in the meal. Electrophoretic analysis of duplicate extracts prepared by the procedure described indicate that the general character of the electrophoretic pattern, the mobilities of the boundaries and the relative amounts of the various components present are reproducible. It is likely, therefore, that the electrophoretic data obtained for the meal extract are representative of the protein pattern of the meal itself. However, the fact that the 2% of the meal nitrogen which is not removed under the conditions of extraction employed may contain additional protein components cannot be overlooked.

*Electrophoretic analysis of arachin:* Arachin was prepared from peanut meal at room temperature essentially as described by Jones and Horn (2). Twelve grams of peanut meal were extracted at room temperature with 4 successive 125-ml. portions of 10% sodium chloride solution and the combined extracts were diluted to 500 ml. The sodium chloride extract contained 97.5% of the protein of the meal and can therefore be considered equivalent to the buffer extract described in the previous section. The precipitate obtained from the sodium chloride extract by adding saturated ammonium sulfate solution to 40% saturation was dissolved in 100 ml. of 10% sodium chloride and reprecipitated in the same manner at 40% saturation with ammonium sulfate. The precipitate was dissolved in a minimum volume of 10% sodium chloride and the arachin was precipitated by dilution with 10 volumes of distilled water. The final precipitate was dissolved in sufficient ammonia buffer to yield a solution containing approximately 0.8% protein and after dialysis against this buffer, was analyzed electrophoretically.

A typical pattern for arachin is shown in Fig. 2. Only two boundaries are visible. Since the mobilities of these two boundaries are - 6.0 and  $-5.3 \times 10^{-5}$ , respectively, they may correspond to the major components A and B of the meal extract pattern. However, it will be noted that a qualitative and quantitative asymmetry exists between the ascending and descending patterns of Fig. 2 that is not apparent in those of Fig. 1. The faster moving major peak on the descending pattern of Fig. 2 appears as a shoulder on the slower moving peak of the ascending pattern. It is probable that this asymmetry is a result

of interaction between the protein components present under these conditions.<sup>6</sup> Such an explanation has been advanced regarding the occurrence of asymmetries in the electrophoretic patterns of other protein mixtures (14, 15, 16). It is evident that the two boundaries do not correspond to independently migrating protein components so that considerable uncertainty is attached to the assignment of relative areas to components A and B in the arachin patterns.

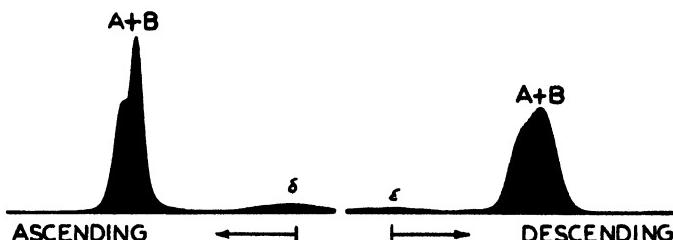


FIG. 2

Electrophoretic Pattern of Twice Reprecipitated Arachin After  
14,820 Seconds in Ammonia Buffer at pH 9.26

A striking feature of the patterns in Fig. 2 is the complete absence from arachin of the minor protein components found to be present in the meal extract. Estimates of the areas under the peaks of Fig. 2 indicate that component A comprises approximately 76% and component B approximately 24% of the total proteins contained in the twice reprecipitated arachin. Due to the high color of ammonia buffer solutions of the first and second arachin precipitates, difficulty was experienced in obtaining satisfactory electrophoretic patterns for these fractions. Sufficient evidence was obtained from the patterns of the crude arachin precipitates, however, to indicate that reprecipitation of arachin does not change either the mobilities or relative amounts of the components present.

<sup>6</sup> It is possible to account for this pattern asymmetry by assuming that two components are present in roughly equal concentrations and that they react to form a dissociable complex as in the fifth type of complex formation considered by Longsworth and MacInnes (14). If this were the case, the fast boundary on the ascending pattern would correspond to component A while the slow boundary would result from the migration of component B plus the complex, only one boundary being present because of the mobile equilibrium between the complex and its components. Similarly on the descending pattern the slow boundary would correspond to component B and the fast one to component A plus the complex.

*Electrophoretic analysis of conarachin:* Conarachin was prepared according to the procedure of Jones and Horn (2). Solid ammonium sulfate was added to the mother-liquor obtained after the precipitation of arachin until the solution was 85% saturated with ammonium sulfate. A portion of the crude precipitate was dissolved in and dialyzed against ammonia buffer (protein concentration, 0.8%) and the electrophoretic pattern was determined (Fig. 3). A second portion of the crude precipitate

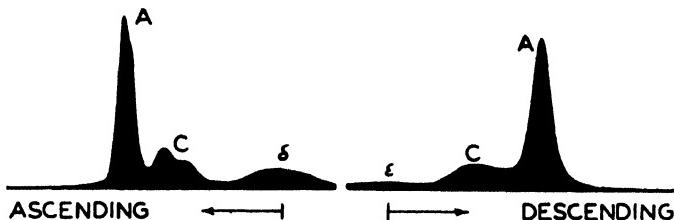


FIG. 3

Electrophoretic Pattern of Conarachin (First Precipitate) After  
15,120 Seconds in Ammonia Buffer at pH 9.26

was dissolved in a minimum volume of 10% sodium chloride solution and the conarachin was reprecipitated by making the solution 85% saturated with ammonium sulfate. The final precipitate was dissolved in and dialyzed against ammonia buffer and the solution (protein concentration, 0.8%) was examined electrophoretically (Fig. 4).

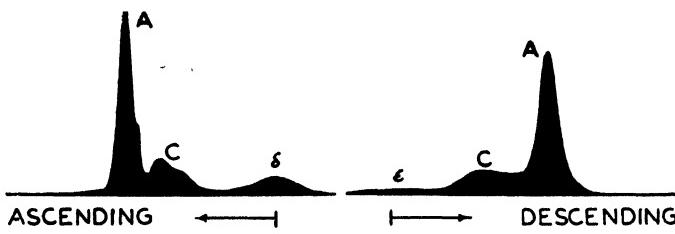


FIG. 4

Electrophoretic Pattern of Conarachin (Second Precipitate) After  
14,400 Seconds in Ammonia Buffer at pH 9.26

The mobilities of the two boundaries visible in the descending patterns of Fig. 3 are  $-6.0$  and  $-3.5 \times 10^{-5}$ , respectively, indicating that conarachin contains component A and the minor components of the meal extract. Protein component B appears to be almost absent from conarachin. No evidence for the presence of component B is apparent in the descending pattern but the presence of a small amount may be indicated by the shoulder on the A peak in the ascending

pattern. Estimation of the relative amounts of the components present in Fig. 3 indicates that component A comprises approximately 80% and the minor components approximately 20% of the conarachin fraction. As is evident from Fig. 4, reprecipitation of the conarachin fraction does not alter significantly either the mobilities of the boundaries ( $-6.0$  and  $-3.4 \times 10^{-6}$ , respectively) or the relative amounts of the components present (A, 78%; minor components, 22%).

It will be observed in Figs. 3 and 4, as mentioned previously, the fraction which appears as a single diffuse boundary in the descending patterns appears to be separated into two boundaries in the ascending patterns.

The mobilities and estimates of the relative amounts of the several components found to be present in the meal extracts, arachin and conarachin, are summarized in Table I.

TABLE I

*Mobilities and Estimates of the Relative Amounts of the Protein Components in the Peanut Meal Extract, Arachin and Conarachin*

Ammonia buffer, 0.1 ionic strength, pH 9.26 at  $25^{\circ}\text{C}.$ ; field strength, 3.1 volts per cm.; protein concentration, approximately 0.8%; mobility in  $\text{cm.}^2 \text{ volt}^{-1} \text{ sec.}^{-1} \times 10^8$  reduced to  $0^{\circ}\text{C}.$ .

Fraction	Protein Component					
	A Mobility	A Per cent <sup>1</sup>	B Mobility	B Per cent <sup>1</sup>	C Mobility	C Per cent <sup>1</sup>
Buffer extract of peanut meal	-6.3	76	-5.1	11	-3.7	13
Arachin, twice reprecipitated <sup>2</sup>	-6.0	76	-5.3	24	—	0
Conarachin, once precipitated	-6.0	80	-	0	-3.5	20
Conarachin, once reprecipitated	-6.0	78	-	0	-3.4	22

<sup>1</sup> Calculated from the descending pattern.

<sup>2</sup> The estimated amounts of components A and B in arachin are open to question due to the asymmetry of the electrophoretic patterns in this instance.

*Approximate distribution of the protein and the major and minor protein components of peanut meal in the meal extract, arachin and conarachin fractions:* The distribution of the protein nitrogen and the amounts of the major and minor components present in the meal extracts, arachin, conarachin, and the conarachin mother-liquor, are given in Table II.

To obtain the figures presented in columns three and four of Table II, 12 g. of peanut meal (No. 35G) were extracted with 10% sodium chloride and the arachin and conarachin fractions were separated exactly as described in the foregoing sections. These fractions were analyzed for nitrogen to determine the distribution of the original meal nitrogen. Since all of the fractions, except No. 1, contained large amounts of ammonium sulfate it was necessary to dialyze each fraction before analysis and to analyze for both total and ammonia nitrogen so that the final figures could be corrected, when necessary, for small amounts of ammonium sulfate that

TABLE II  
*Approximate Distribution of the Protein Components in the Peanut Meal Extract, Arachin and Conarachin*

No.	Fraction Description	Protein Nitrogen Per cent of meal nitrogen	Protein <sup>1</sup> g.	Components Major g.	Components Minor g.
1	Buffer extract of peanut meal	97.5	5.44	4.73	0.71
2	Arachin, first precipitate	63.8	3.56	3.56	0.0
3	Arachin, second precipitate	62.4	3.48	3.48	0.0
4	Arachin, third precipitate	56.8	3.17	3.17	0.0
5	Conarachin, first precipitate	33.2	1.85	1.48	0.37
6	Conarachin, second precipitate	32.9	1.84	1.43	0.41
7	Conarachin mother-liquor	2.9	0.164	—	—

<sup>1</sup> Protein = protein nitrogen  $\times$  5.5; yields based on 12 g. meal used.

were not removed by dialysis. The reprecipitated arachin and conarachin fractions (Nos. 4 and 6 of Table II) and the thoroughly dialyzed (100 hours) conarachin mother-liquor (No. 7 of Table II) were lyophilized (17) to yield dry preparations weighing 3.0, 1.43 and 0.13 gms., respectively.<sup>7</sup> The weights of the protein fractions given in Column 4 (Table II) were calculated by assuming that the nitrogen factor 5.5 (2) was applicable to all fractions.

The figures given in the last two columns of Table II were obtained by multiplying the figures given for the percentage composition of each of the fractions (Table I) by the weight of protein in the fraction (Table II). As was pointed out above, it is impossible to assign accurate values for the relative amounts of components A and B in arachin due to the asymmetry which was found to exist in the electrophoretic patterns for arachin. Consequently, in Table II no attempt has been made to indicate the distribution of the A and B components separately but the two have been grouped as major components. This treatment is valid and satisfactory for the purposes of the discussion to follow.

<sup>7</sup> Owing to the difficulty of obtaining quantitative recovery in the lyophilization process it is to be expected that the weights of the isolated fractions would be lower than those calculated from the protein nitrogen content of the corresponding fractions.

Concerning the results given in columns three and four of Table II the following points are noteworthy. Arachin and conarachin are present in the meal in the ratio of approximately 2:1 and together they constitute approximately 97% of the total protein of the meal. These figures differ from those obtained by Jones and Horn (2) who found arachin and conarachin to be present in the ratio of approximately 3:1. It should be noted in this connection, however, that the sodium chloride extract, prepared under the conditions described in this paper, contained 97.5% of the protein nitrogen of the meal while the corresponding extract of Jones and Horn contained only 87% of the total meal nitrogen.

Obviously, the total protein nitrogen or protein contained in fractions 2, 5 and 7 (Table II) should equal that contained in the meal extract. According to the figures given in the table, the total protein nitrogen or protein contained in these three fractions slightly exceeds that for the meal extract, but when the number of operations required to obtain the analytical figures is considered the agreement may be regarded as satisfactory.

Comparison of the values for fractions 2, 3 and 4 (Table II) reveals that reprecipitation of the first arachin precipitate from 10% sodium chloride solution at 40% saturation with ammonium sulfate, results in only a slight loss of protein, while in the second reprecipitation of arachin by dilution of a 10% sodium chloride solution of the protein appreciable loss of protein occurs. Reprecipitation of conarachin (Nos. 5 and 6, Table II) can be accomplished with only a negligible loss of protein.

Concerning the results presented in the last two columns of Table II it is apparent that the sum of the weights of the major and minor components present in Fractions 2, 5 and 7 should equal the weight of these components originally present in the meal extract. In the case of the major components the agreement is satisfactory and it may be concluded tentatively that all of the major components are accounted for in arachin and conarachin. However, a considerable amount of the minor components is not accounted for in either arachin or conarachin. Since the minor components are absent from arachin and appear in the conarachin fraction it is evident that the minor components are completely soluble under the conditions used for the precipitation of arachin. It is apparent also, on the basis of the electro-phoretic data, that the minor components are sufficiently soluble even

under the conditions used for the precipitation of the conarachin fraction to permit approximately one-half of the minor components to remain in the final mother-liquor.

It may be concluded from the results recorded in Table II that arachin comprises approximately 63% of the protein of the meal and that it consists entirely of the major protein components A and B. Arachin contains none of the minor components. Conarachin comprises approximately 33% of the protein of peanut meal and it consists of approximately 80% of component A and 20% of the minor components. Conarachin appears to contain none of the B component. The protein fraction<sup>8</sup> which remains after the removal of arachin and conarachin from the meal extract appears to consist only of the minor components and comprises approximately 3% of the protein of the meal.

*Chemical composition of the protein fractions of peanut meal:* On the basis of the conclusions reached in the preceding section, certain inferences can be drawn with respect to the chemical composition of the several protein components of the peanut. The whole protein of peanut meal contains approximately 0.5% sulfur (1) while arachin contains 0.4–0.52% and conarachin 1.09–1.22% sulfur on an ash-free basis (1, 4). Since component B is absent from conarachin, and therefore cannot be responsible for the high sulfur content of the conarachin fraction, this extra sulfur must be present in either component A or in the minor components. However, even if the implausible assumption is made that component B contains no sulfur and that consequently all of the sulfur of arachin is present in component A, then component A would still possess a sulfur content of less than 1 % which is appreciably lower than that found for conarachin. It may be concluded, therefore, that the major part of the extra sulfur of conarachin must be present in the minor components. On this basis the minor components could contain approximately 3% sulfur. An opportunity to verify this deduction was provided since the protein which remains in

<sup>8</sup>The composition of this fraction and the discussion concerning it which follows are based exclusively on the data given in Tables II and III. The deductions made were not verified by electrophoretic analysis of this fraction since its importance and interesting nature were not fully appreciated until the experimental results were organized for publication, at a time when the materials and equipment necessary for continuing these investigations were no longer available to two of the authors (G.W.I. and T.D.F.). Therefore, the conclusions made regarding this fraction must be considered as tentative pending direct experimental verification.

the mother-liquor after the precipitation of conarachin has been shown by consideration of the electrophoretic results to be composed primarily, if not exclusively, of the minor components. The sulfur content of this protein should, therefore, be quite high.

That this is true is illustrated in Table III. Arachin and conarachin were prepared exactly as described above for the preparation of these fractions for electrophoretic analysis. In each case the moist protein precipitate (arachin was reprecipitated twice, conarachin once) was

TABLE III  
*Analysis of Peanut Protein Fractions<sup>1</sup>*

Fraction	Total Nitrogen per cent	Ash per cent	Total Sulfur per cent	Inorganic Sulfur per cent	Organic Sulfur per cent
Arachin	16.6	0.47	0.44	0.07	0.37
Conarachin, light-colored fraction <sup>2,3</sup>		0.92	1.10	0.13	0.97
Conarachin, dark-colored fraction <sup>2</sup>	18.2	0.48	1.80	0.13	1.67
Protein from conarachin mother-liquor	14.3	9.29 <sup>4</sup>	3.04	0.14	2.90

<sup>1</sup> Analyses are reported on a moisture-free basis.

<sup>2</sup> The organic sulfur value for the composite conarachin fraction calculated from the values given in the table for the light- and dark-colored fractions is 1.34%.

<sup>3</sup> This sample possessed a high electrostatic charge which made duplication of analyses upon the small amount of sample available extremely difficult.

<sup>4</sup> The high ash content of this sample is due to the concentration of the bulk of the non-dialyzable ash of the peanut meal in this fraction.

suspended in water in a cellophane membrane and dialyzed for 72 hours against running distilled water in a rocking dialyzer after which the contents of the cellophane membrane were transferred to a flask and lyophilized to yield a dry preparation. The mother liquor which remained after precipitation of the conarachin fraction was also dialyzed against running distilled water for 72 hours, and the solution remaining in the membrane was lyophilized to dryness. The dry material was redissolved in a minimum volume of water, redialyzed for 72 hours against running distilled water and finally lyophilized. This precaution was taken to insure the complete removal of ammonium sulfate. The lyophilized preparations were dried *in vacuo* for 18 hours at 50°C. for analysis.

During the preparation of the conarachin sample it was observed that the material in the membrane after dialysis consisted of two

distinct fractions: one was a dark-colored, gummy, insoluble fraction; the other was light in color and appeared to be quite soluble. Since, after lyophilization, these two fractions could be separated quite readily both the light-colored (570 mg.) and dark-colored (660 mg.) fractions were analyzed separately.

It is apparent from the results presented in Table III that the protein which remains in the mother-liquor after the removal of the arachin and conarachin fractions has a very high sulfur content, confirming the conclusions drawn above from a consideration of the electrophoretic data and published analytical figures that the minor protein components of the peanut must be high in sulfur.

The organic sulfur values for the arachin fraction and for the composite conarachin fraction correspond closely with those reported in the literature. It is interesting to observe, however, that the light- and dark-colored conarachin fractions differ markedly in composition. It is apparent that fractionation of conarachin takes place during dialysis and that the dark fraction contains relatively more of the sulfur-rich protein components than does the light-colored fraction.

## DISCUSSION

Johns and Jones (3) found that the cystine content of conarachin was higher than that for arachin. The more recent work of Brown (4) has confirmed this observation and has shown further that an even greater difference exists with respect to the methionine contents of the two fractions. Brown's figures indicate that arachin contains 1.51% cystine and 0.67% methionine as compared with 2.92% cystine and 2.12% methionine in conarachin.<sup>9</sup> From what has been presented in the preceding section it is probable that the minor protein components of peanut meal may be particularly high in both cystine and methionine.

The results of Brown (4) for the hydroxyamino acids of arachin and conarachin raise another interesting possibility. Conarachin appears to contain approximately 1% of hydroxyamino acids other than serine and threonine, while all of the hydroxyamino acids of arachin are accounted for by serine and threonine. Since, according to these results and the results reported in this paper, neither the A nor the B components of peanut meal can contain any hydroxyamino acids, other than serine and threonine, it is possible that the minor compo-

<sup>9</sup> Baernstein (7) has found arachin to contain 1.33% cystine and 0.54% methionine.

nents of the peanut may be particularly high (as much as 4%) in hydroxyamino acids other than serine and threonine.

If subsequent work should prove the validity of the supposition advanced above, namely, that the minor protein constituents of the peanut may be rich in the essential amino acid methionine, several points regarding the nutritional value of the peanut and of the arachin and conarachin fractions become apparent. Baernstein (7) has found that whole peanut protein is of high quality nutritionally and that it is approximately equivalent to casein in its ability to support growth of the white rat. For the rat, conarachin alone is an adequate protein. Arachin alone is deficient but may be supplemented by conarachin to produce a nutritionally adequate protein. These facts, together with the evidence of White and Beach (18) that arachin supports growth when supplemented with methionine (or homocystine) makes it apparent that the adequacy of peanut protein or any of its fractions may depend primarily upon the amount of the methionine-rich minor constituents present in the fraction fed. This becomes important when it is considered that the minor components appear to be the most soluble of the protein components of the peanut under these conditions of fractionation and that, consequently, these components are most likely to remain in the final mother-liquor. This has been demonstrated above in the preparation of arachin and conarachin and may account for the report of Sure (19) who found that conarachin did not supplement arachin nutritionally whereas whole peanut protein was adequate. It is conceivable that Sure's preparation of conarachin may have contained even less of the minor components than the preparation which we have analyzed or the preparation which White used in his experiments.

In view of the fact that protein component A is common to both arachin and conarachin, the sharp and complete precipitation of the arachin fraction in a solution which is 40% saturated with ammonium sulfate is interesting. It is possible that this represents an example of coprecipitation of two protein components in which A and B precipitate together from 40% ammonium sulfate in the approximate ratio of 3:1 as long as any of component B is present in the solution. In the absence of component B, component A is soluble in 40% ammonium sulfate and can be completely precipitated only when the ammonium sulfate concentration is raised to 85% of saturation.

Eirich and Rideal (20) have reported the ultracentrifugal analysis

of "a globulin arachin from the ground nut." Unfortunately, the method of preparation of the protein used is not described and it is uncertain whether it corresponds to the arachin of Johns and Jones or represents the total globulin of the peanut. It is interesting to note that in 0.1 *N* ammonia solutions (conditions which closely approximate those used in the electrophoresis experiments reported in this paper) their protein exhibited the three molecular weight groups 400,000, 140,000 and 20,000. The more recent results of Campbell and Johnson (21), however, indicate a molecular weight of approximately 250,000 for a "nearly homogeneous globulin (obtained from ground nut)." Measurements were made in the ultracentrifuge upon a solution containing 0.5 g. of the protein per 100 ml. and having a pH of 8.0.

Although tentative conclusions have been reached that the peanut contains at least three and probably four protein components, and that arachin and conarachin each consist of at least two protein components, no evidence is afforded by the results of the present investigation to indicate whether these components are simple proteins or whether one or more of the components might represent a combination of protein with carbohydrate, lipid or other substance. In oilseeds such as the peanut, ample opportunity exists for the formation of glyco- and lipo-proteins and some evidence, as yet unpublished, has been obtained to indicate that both of these classes, and possibly other conjugated proteins, may exist in the peanut.

The authors wish to express their appreciation to the Analytical Sections of both the Southern and Eastern Regional Research Laboratories for carrying out the analyses reported in Table III.

#### SUMMARY

Electrophoretic analysis of an extract containing at least 98% of the protein of peanut meal indicates the presence of two major protein components which occur in the approximate ratio of 7 to 1, and which, together, comprise approximately 87% of the protein in the extract. The remaining 13% of the protein in the extract probably consists of at least two minor protein components present in approximately equal amounts.

Arachin, which comprises approximately 63% of the protein of peanut meal, has been found to consist only of the two major protein components, the proportions being approximately 76% of the electro-

phoretically faster moving component (A) and approximately 24% of the slower moving component (B). Arachin contains none of the minor protein components of the meal.

Conarachin, which comprises approximately 33% of the protein of peanut meal, has been found to consist of approximately 80% of component A and 20% of the minor components. Conarachin appears to contain none of the B component of peanut meal.

The protein which remains after the successive removal of arachin and conarachin from a peanut meal extract consists largely, if not exclusively, of the minor protein components and comprises approximately 3% of the protein of the meal. This protein fraction has been isolated and has been found to contain 2.9% sulfur, a particularly high sulfur content for a vegetable protein. Deductions made from the results presented and from published analytical data indicate that the high-sulfur fraction may contain relatively large amounts of methionine and, possibly, large amounts of hydroxyamino acids other than serine and threonine.

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## Book Reviews

**Optical Activity and Living Matter.** By GEORGE FRANCIS GAUSE, Professor of Experimental Biology, University of Moscow. Normandy, Mo., Biodynamica, 162 p., 1941.

It has been known for more than a century that stereochemical orientation is a dominant attribute of living matter. Gause, who deserves special merit for his work on the biology of spirally twisted organisms, differentiates between dissymmetry as a property of individual components of a system and asymmetry consisting of an aggregate of molecules. (Because of Pasteur's influence, however, another point of view omitting this differentiation prevails throughout the literature.) In his book, Gause develops the theory that dissymmetry is a characteristic property of the basic components of protoplasm, while the actual metabolic products, *e.g.*, urea, uric acid, creatinine, and hippuric acid are optically inactive. The following facts, however, negate this generalization: glycine, a protein degradation product, is optically inactive, whereas other common products of metabolism such as combined glucuronic acids, lactic acid, hydroxyproteic acids, uroferric acid, uric dextrins (animal gum), biliary pigments and bile acids (in feces), serine (in sweat), and most of the vitamins and hormones are optically active. Allantoin is probably optically inactive only because of a keto-enol-tautomerism. Such a differentiation is even less conceivable in plant metabolism.

The author also mentions that asymmetric inorganic structures exist. Their strong optical activity which, of course, only applies to solid crystals, should be considered. A more detailed report would have been desirable on the important experiment of Pope and Kipping who found that the ratio of the sodium chlorate antipodes crystallizing from a glucose solution is unequal. This cannot be explained only as a statistical mean value indicating deviations. Tin might have been mentioned as one of the elements functioning as the asymmetric nucleus of optically active compounds. The same holds for compounds of cobalt, *e.g.*, the dodecammine-hexol-tetracobaltic bromides, which are strongly optically active in aqueous solution and do not contain carbon in complex coordination, but are purely inorganic. It is regrettable that the above mentioned fact is never discussed in similar considerations. The author cites a contention which would be of general interest, if correct, that malic acid is originally optically active but becomes racemized in some plants when it assumes the character, as it were, of a storage material. This contention, originating with Ruhland and Wetzel, has been strongly contested by Bennet-Clark. The interpretation which connects racemization with the alleged storage function is wrong in any event. Investigations of Pederson, Peterson, and Fred on the various forms of lactic acid show that racemization may be produced by any enzyme showing the same affinity to both antipodes of lactic acid and forming an enzyme substrate complex which allows the reversible formation of structurally inactive intermediary

components. The assumption of a specific racemase thus becomes superfluous, and it becomes plausible that occasionally postmortally formed lactic acid is found to be inactive.

The much emphasized principle of biological series, also mentioned by Gause, postulated that all natural amino acids belonged to the *l*-series. This principle was disproved in some respects in 1936 by the discovery of *d*-threonine, which enters into the architecture of ordinary proteins. The fact that petroleum, according to its local origin, displays varying and opposite optical activity points to the possibility that quite different organic materials participated in the formation of the respective varieties of oil during the geological periods, and that one cannot consider cholesterol alone, as has been done previously, as the sole source of optically active compounds. The assumption of W. Kuhn as to whether differences in solubility between "*d*- and *l*-amygdalin" can be decisive for the occurrence of the gentiobiosides of *d*- and *l*-mandelonitrile in various parts of a plant, remains questionable. It may be mentioned that the now numerous investigations of the optical resolution of racemates in mammalian metabolism, which started with the research on *d,l*-carbohydrates, and *d,l*-amino acids in 1901 and 1905, respectively, have been continued since 1906 by Abderhalden and others. The statement that quinine and quinidine are optical antipodes is an error found in many treatises on pharmacology (the two alkaloids are in reality isomers of different melting point, different solubility, and numerically different optical activity); their interrelation is not analogous to that of *d*- and *l*-glucose but rather to that of *d*-glucose and *l*-mannose. This may be emphasized in view of the present problem of malaria therapy. The unexplained, important observations on the existence of stereospecifically selective esterases in the organs of a single species are duly stressed.

The asymmetric syntheses *in vitro* by Marckwald and McKenzie are reported. The well-known theories concerning the primary origin of optical activity in organic substances are excellently discussed, and the last phase of this development, the photochemical experiments and statements of W. Kuhn, are clearly expressed.

The disquisition of the author on the subject of spiral twist and optical activity are especially stimulating. As far as the reviewer can judge it, an equally instructive and complete survey of this phenomenon, which had already engaged the mind of Leonardo da Vinci, cannot be found anywhere. Forms of dextral and sinistral micro-organisms are illustrated, and the conditions of their appearance and their constancy in nature are described. There exist a few observations that the usual twist in molluscs is determined by a dominant gene while the inverse forms are controlled by a recessive gene; nevertheless, the conditions for the appearance of such mutations with morphological inversion are rather obscure. Gause adduces several interesting experimental contributions supporting the suggestion that "secondary substances" of metabolism are optically inverted and thus may bring about morphological inversion, whereas the protoplasm of dextral and sinistral forms is identical. The author develops a principle of asymmetric analysis. This consists primarily in the examination of relative toxicity of chemical antipodes and of their influence on the evolution of the nervous system, and on the mechanism of some physiological functions in lower organisms. A chapter on the alleged occurrence of unnatural amino acids in cancer tissue concludes the book. In reference to the negative criticism of Koegl's experiments one must point to the last remark of Koegl: It may not be the problem whether

d-amino acids occur in malignant tumors, but whether there exist in tumors enolizable peptide linkages susceptible to racemization.

The present monograph offers information and stimulation for all those who are interested in the general significance of the asymmetry problem in animate nature. Other aspects in this field, which have not been treated in this book, can be found in a publication of McKenzie, *Ergeb. Enzymforsch.* 5, 49 (1936), and in an article of Neuberg and Lustig: "Pancreas and the Problem of Asymmetry," *J. Expl. Med. Surgery* 1, 313 (1943).

C. NEUBERG, New York, N. Y.

**The Avitaminoses.** By WALTER H. EDDY, Ph.D. and GILBERT DALLDORF, M.D. Third edition. The Williams & Wilkins Company, 1944, pp. 438

The teamwork of a biochemist and a pathologist has produced a timely, comprehensive, fairly critical, and hence reliable monograph for the guidance of physicians, biochemists, and other investigators and teachers in the field of foods and nutrition.

The volume is divided into three main sections: I. The Vitamins, their chemistry, occurrence in nature and their known action in the animal body (pp. 3-125). II. The avitaminoses, the known, the probable, and suspected effects of deficient ingestion of the vitamins (pp. 129-352). III. Technical methods of vitamin assay, and vitamin assay of food (pp. 355-378).

The section on the avitaminoses, which takes up more than half of the book, is particularly valuable in its generally comprehensive and critical appraisal of the pressing and difficult problems of subclinical vitamin deficiencies and the numerous fears and foibles (if not worse) about vitamin cure-alls for nearly all human ailments and physical and mental deficiencies of man today. In view of the general scientific attitude of the authors, it is perhaps, ungracious to point out occasional failures in mental alertness. In the preface the authors refer to "the significance of the vitamins as disease provokers." This is, to say the least, confusing, since it is not the excess but the deficiencies in vitamins which lead to impairment of health. We are told (p. 173) that "thiamin deficiency is very common." On the basis of existing evidence, this appears both confusing and misleading. In the chapter on vitamin requirement of man (pp. 53-60) they introduce the three page table of Jolliffe and Most, outlining the vitamin deficiency symptoms in man, "that permit diagnosis of vitamin deficiency." This is an unfortunate lapse in an otherwise excellent monograph. Drs. Eddy and Dalldorf (and other informed biologists) know that most of the signs and symptoms listed in that table are not specific for vitamin deficiencies.

A. J. CARLSON, Chicago, Ill.

**Manometric Techniques and Related Methods for the Study of Tissue Metabolism.** By W. W. UMBREIT, R. H. BURRIS, and J. F. STAUFFER. First Edition. \$3.50. Pp. 198. Burgess Publishing Company, 426 South Sixth Street, Minneapolis, Minnesota. 1945.

This volume is the cooperative effort of a group of staff members of the University of Wisconsin. The authors state that the book is to be regarded as a laboratory companion to the volume on respiratory enzymes published several years ago by a similar group of Wisconsin investigators and is designed to provide a workable

series of methods for the use and instruction of graduate students in the general field of tissue metabolism.

Chapters I, II, III, IV, VI, VIII and XI contain descriptions and directions for the manipulation of various types of manometric equipment. The subject matter is similar to that discussed in Dixon's admirable book on "Manometric Methods." However, the treatment is somewhat more elaborate and is illustrated by frequent sample calculations. In particular, Chapter III contains a useful chart, not available elsewhere, showing the relationship between bicarbonate concentrations and pH and the generalized treatment in Chapter IV of the theory of the indirect Warburg method deserves favorable comment.

Chapter V contains a compilation of detailed procedures for confronting various technical difficulties that arise in the course of manometric studies. It is the sort of information that is usually transmitted from one investigator to another verbally and to the reviewer's knowledge has not been summarized so conveniently in any other place.

Chapters VIII and IX are concerned with methods for preparing tissues for investigation and represents a documented survey of procedures previously available only in isolated original articles in the literature.

Chapter X contains a selection of chemical methods on the determination of the more common tissue metabolites. In view of the variety of methods available for many of these substances it is to be expected that there might be disagreement with the selection of particular methods in the list. For example, in the reviewer's laboratory Friedmann's method for pyruvic acid and Nelson's modification of Somogyi's method for glucose have been found to be preferred to those described in the text.

Chapter XII is to be recommended for its description of an improved technique for the estimation of dehydrogenase activity by the Thunberg method.

Chapter XIII is concerned with electrometric techniques and constitutes a very brief survey of a large field.

Chapters XIV, XV, and XVI, concerned with manometric methods for the estimation of metabolites and enzyme systems, methods for the analysis of phosphorylated intermediates and methods for the preparation of physiologically important intermediates and metabolites, are of the greatest convenience and usefulness. They justify in themselves the publication of the entire volume.

As one might expect, there are also matters for criticism. It does not seem desirable to define fermentation as any "transformation which occurs in living cells and which does not employ gaseous oxygen (page 8)," and it would be helpful, if in Chapter XVI, it were clearly stated that the calculations and conversion factors referred to the free acids concerned (*i.e.* the term hexose-diphosphate is used and the calculations are based on hexose-diphosphoric acid) or if they were expressed entirely on a molar basis.

However, the book constitutes a very helpful reference and laboratory aid and its purchase is to be recommended to investigators in the field of tissue metabolism and to those who are anxious to acquaint themselves with the techniques of this field.

E. A. EVANS, JR., Chicago, Ill.

**Chemistry and Technology of Food and Food Products, Volume I.** Edited by M. B. JACOBS. Interscience Publishers, Inc., New York, 1944. 903 pp. Price \$10.50.

The treatise edited by Jacobs is divided into two volumes, the first on the chemistry of foods, the second on the technology of foods. Volume I, which is reviewed here, is itself divided into two parts. Part One, which takes up 380 of the 903 pages, deals with general biochemical subjects such as carbohydrates, proteins, enzymes, and vitamins. It adds considerably to the bulk of the volume and will be useful only to those without convenient access to standard books on biochemistry. The subjects, for the most part, are treated as in the ordinary books on biochemistry, with no particular emphasis on their special relations to the problems of food chemistry.

Part Two of the book consists of discussions of individual foods and food products by experienced specialists. The subjects and authors are as follows:

- Milk, Cream, and Dairy Products—Jacobs.
- Meat and Meat Products—Urbain.
- Fish, Shellfish, and Crustacea—Stansby.
- Poultry and Eggs—Pennington.
- Edible Oils and Fats—Bailey.
- Cereal Grains—Geddes.
- Baking and Bakery Products—Cathcart.
- Vegetables, Mushrooms, Nuts, and Fruits—Lee.
- Carbohydrate and Sugar Foods—Degering.
- Confectionery and Cacao Products—Schoen.
- Coffee and Tea—Ukers.
- Flavors, Spices, and Condiments—Worrell

The subjects are extremely different from one another, and hence it would be impossible to have them treated adequately by a single author. The general literature of food chemistry being poor, this gathering together in one convenient volume of much varied information, will certainly be very useful.

Despite the length of the book, the subject of food chemistry is so large, that the discussions of the separate foods are still very elementary, although very frequently peculiarly detailed facts are given which are of interest only to the expert.

Actually, a very large part of each section is devoted not to chemistry, but necessarily to a quite non-chemical description of the different foods and how they are handled. Indeed, it is astounding how little chemical knowledge there is of foodstuffs which are produced in enormous volume.

The food industry, with some notable exceptions, is only now beginning to devote itself to chemical research in the intensive manner common in the chemical industry. It is to be expected that in the future, a considerable fraction of all biochemists will be occupied with the problems of food chemistry, that the curricula of the universities will have to be adjusted to this fact, and that the volume of Jacobs is a sign of a very much expanded literature of the chemistry of foods.

**The Chemistry and Technology of Food and Food Products.** Volume II. Edited by M. B. JACOBS, Interscience Publishers, Inc., New York, N. Y. 890 pages, 25 chapters, \$10.50 (\$19.00 for volumes I and II).

Written by 27 scientists and food technologists, several of whom are well known specialists in food research and processing; although among them are also several names new to this reviewer. The 25 chapters are divided into four parts, namely, "unit operations and processes," "sanitary and quality control," "preservation" and "production." In general, the sequence of subjects is similar to that of Volume I, that is the earlier chapters deal with more or less basic and general principles and considerations, and the later ones with the technology of various industries.

An attempt is made to separate "food preservation" from "production." In this reviewer's estimation such separation is very difficult and is artificial, and apt to lead to repetition. Thus, the chapters on dehydration of foods, refrigeration, canning and pickling (preservation by microorganisms) certainly deal with food production (technology), as well as with food preservation, although they are placed in part V (preservation).

In most food industries both preservation and production are involved and interdependent. On the other hand chapters such as those on "unit operations," "packaging" and "washing," treat of well defined concepts or processes. Incidentally, we are pleased to note the emphasis placed on "unit operations" and "processes" as their consideration unifies and provides a sound foundation for discussion of food preservation and production.

It appears to this reviewer that more space than necessary has been given in the chapters on unit operations and processes to descriptive matter not particularly pertinent to the main topics under consideration. For example, the description of various kinds of dryers might better have been presented in the chapter IX on dehydration; and much of that on humidity, atmosphere, etc. in relation to refrigeration, in chapter X. In chapter V on food machines dehydration and refrigeration are again considered and some of the unit operations of chapters I and II are again presented. Nevertheless, these three chapters are very useful.

As in Volume I the chapters on the technology of the several industries are necessarily all too brief because of space limitations; for example 27 pages only for "confectionery and cacao products" and 31 for "milk and milk products" which we suppose is one of the penalties an editor must pay when so many and such diverse subjects are treated in one volume. The writers are to be complimented on condensing their subjects so well.

Some chapters contain a goodly number of citations to the literature; others are somewhat lacking in that respect.

On the whole this volume will be found very interesting and useful to food technologists, teachers and students. In spite of the criticisms made by this reviewer, it brings together in readily consulted form much of the knowledge of food chemistry and technology.

W. V. C'RUESS, Berkeley, Cal.

**Advances in Enzymology**, Volume V, edited by F. F. NORD and C. H. WERKMAN. Interscience Publishers, Inc., New York, 1945. vii + 268 pp. Price \$5.50.

The present volume contains a cumulative subject index of the first five volumes of this important publication. A perusal of the index shows the wide variety of subjects in the enzyme field which have already been covered in previous volumes, but in this rapidly expanding field there is no dearth of new topics. Articles, such as those found in the present volume, are of distinct aid to workers engaged in enzyme research, because even the specialist finds it difficult to keep track of all new developments or to locate some significant observation that has been made in the past.

The subjects covered in volume V, all of which cannot be adequately reviewed here, concern the properties of plant viruses, coagulation of the blood, amino acid decarboxylases, fermentation of oligosaccharides, pyruvate metabolism, biochemistry of Fusaria, nicotinamide, and enzyme reactions of sulfur compounds.

In the article of Pirie on plant viruses one is impressed by the large amount of well-substantiated knowledge concerning the physical and chemical properties of this unique class of nucleoproteins. As pointed out by the author, no other protein, with the exception of hemoglobin, has been studied so thoroughly and by so many different methods. Because of the relation of the virus problem to a number of important biological problems, such as gene action, self-reproduction and malignancy, no effort is being spared to advance this field. So far no definite enzyme activity has been found in the virus particles.

Chargaff's article on blood coagulation is helpful in its critical evaluation of facts, often based on the author's own experience. This is an accomplishment by no means easy in a subject to which more than 2000 papers have been contributed. Progress is definitely connected with the often difficult and laborious purification of the different components of the system. The two steps in the process of blood coagulation which may be enzymatic are the conversion of prothrombin to thrombin and the action of the latter on fibrinogen. Although it is stated in the article "that the groupings in fibrinogen that are attacked by thrombin are completely a matter of conjecture" it is noteworthy that an article has since been published (Lyons, *Nature* 155, 633 (1945)) in which a suggestion by Baumberger appears to be supported, namely, that fibrin is formed as the result of S-S bridges produced by the oxidation of SH groups of fibrinogen by thrombin, a process in which vitamin K acts as the oxidant.

Leibowitz and Hestrin have summarized the existing knowledge of disaccharide fermentation in yeast. They have built up a strong case for the view that maltose and other disaccharides can be fermented directly, i.e. without preliminary splitting by hydrolytic enzymes. Although this direct fermentation could be explained by a phosphorolytic splitting of the disaccharides, they point out that nonhydrolytic enzymes, such as sucrose phosphorylase and levansucrase which are found in bacteria, have so far not been found in yeast. In a discussion of the metabolic regulation in living cells they take the view that enzymatic processes of cell extracts disclose potential mechanisms, but not necessarily mechanisms operative in the living organism.

The review of Stotz stresses the importance of the determination of blood pyruvate levels in intact animals under different experimental conditions, and it is stated by

the author that the under-utilization theory of diabetes gains considerable support from the nature of the pyruvate response to glucose and insulin in diabetes. The many channels of pyruvate metabolism as well as the interrelations with protein and fat metabolism are presented in a clear and concise manner and are illustrated by well-chosen schemes.

Nord and Mull have summarized the literature on the biochemistry of *Fusaria* which appeared since 1938. These organisms can reduce nitrate to nitrite, a reaction which is coupled with the formation of pyruvic acid from hexoses and pentoses, apparently without the intermediate formation of phosphoglyceric acid. The great versatility of these organisms is also shown by the fact that they can use elementary sulfur as hydrogen acceptor, resulting in the formation of  $H_2S$ .

In the article of Schlenk some 20 reactions are listed which are catalyzed by specific dehydrogenases in combination with either coenzymes I or II. The inhibitory effect of decomposition products of butter yellow, a carcinogenic azo-compound, on triose phosphate dehydrogenase and other enzymes is discussed; increasing concentrations of coenzyme I counteract the inhibition on triose phosphate dehydrogenase. The recent literature on the enzymatic decomposition of coenzymes I and II is summarized, a field which is gaining increasing importance.

Smythe has reviewed the enzymatic transformation of cysteine to pyruvic acid and the enzymatic hydrolysis of glutathione.

CARL F. CORI, St. Louis, Missouri

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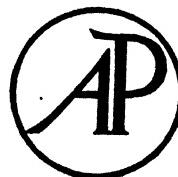
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VOLUME 8



1945

ACADEMIC PRESS INC. PUBLISHERS  
NEW YORK, N. Y.

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# On the Possible Presence of Alloxan in Normal Animal Tissues and Fluids<sup>1</sup>

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Received May 26, 1945

## INTRODUCTION

Since alloxan is able to induce experimental diabetes in animals, it has naturally been surmised that it may be connected with the cause of human diabetes mellitus. It therefore was of interest to examine the tissues and fluids of normal animals for its possible presence. To date, the only such observations, made many years ago, appear to be its detection in the gelatinous mucus from an intestinal catarrh (2) and (as murexide) in the urine of a patient (3) suffering from heart disease.

Many color tests for the identification of alloxan and its reduction products (alloxantin and dialuric acid) have been reported. Unfortunately, in the forms described in the literature, these tests are not well suited to the study of biological materials and only a few, if any, of them appear to be truly specific.<sup>2</sup> We have therefore felt that a minimum of two tests would be necessary to establish with any degree of certainty the presence or absence of alloxan or its reduction products in a material under examination. Consequently, we selected two of these tests and have modified them for use in the study of a variety of animal tissues and fluids. The first of these, the pyrrole test, is given by both alloxan and dehydroascorbic acid (6); the second, the "purple" test, is given by alloxan, ninhydrin and their reduction products. If an "unknown" material is found to respond to both tests it seems reasonable to conclude that it may contain alloxan or its reduction products.

<sup>1</sup> A note on a part of the present study has been published (1).

<sup>2</sup> However, after the initiation of the present study, Archibald (4) described several excellent methods of estimation; and, since our manuscript was submitted, a valuable microbiological and fluorimetric test has appeared (5).

*A Modification of Agrestini's Pyrrole Test for Alloxan*

Agrestini (7) has described a color test for alloxan which consists in boiling 2 cc. of a solution of the substance with 1 drop of pyrrole, resulting in a violet-blue color which changes to red on cooling; addition of sodium hydroxide solution then gives a green color which changes to an intense blue.

This test is obviously of little or no value when applied, in the form described by him, to animal extracts containing proteins and other substances precipitated by heating. However, we find that trichloroacetic acid, introduced by Greenwald (8) as a protein-precipitant, may be advantageously employed without affecting the formation of a color (blue) on adding pyrrole and heating, preferably to 50°. The presence of one part of alloxan hydrate in 50,000 parts of solution may thus be readily detected by the naked eye. An additional advantage in the use of trichloroacetic acid is that, in appropriate concentration, the resultant extract has a pH at which alloxan appears to be stable for many days.

A fainter blue color is given by alloxantin; if the solution in 5% trichloroacetic acid is shaken with charcoal (which facilitates oxidation of alloxantin to alloxan) before performing the test, the rich blue color caused by alloxan is observed.

*The Pyrrole Test* (6). To 5 cc. of a solution (*e.g.*, 0.01%) of alloxan hydrate in 5% aqueous trichloroacetic acid was added 0.02 cc. of pyrrole (*or*, for qualitative tests, 1 drop of pyrrole), the suspension gently agitated for a few seconds until the pyrrole had dissolved, and the solution kept in a thermostatically-controlled bath at 50° for five minutes. The time required for a blue color to develop (and its intensity)

- depends on the concentration; for very dilute solutions (less than 0.002%) the time of heating may be extended to 20 minutes.

When 5 cc. of an 0.01% alloxan hydrate solution in 5% trichloroacetic acid was treated as above and then kept at room temperature, a blue precipitate gradually settled from the blue solution during the course of a few hours. As with dehydroascorbic acid, the formation of this precipitate was considerably delayed if one-tenth the above amount of pyrrole was employed for testing the *dilute solutions*: when 0.002 cc. of pyrrole was added to 0.002% alloxan hydrate and the solution heated at 50°, the resultant blue color displayed a band at 620–625 m $\mu$ . The optical densities were measured<sup>3</sup> as previously described (6), on a Beckmann quartz spectrophotometer.<sup>4</sup> At this concentration (0.002% alloxan monohydrate), the optical densities in the range 600–650 m $\mu$  were close to those of the blue color given by fresh 0.01% dehydroascorbic acid solution under the same conditions.

<sup>3</sup> This determination was kindly performed by Miss Jacqueline Front.

<sup>4</sup> We are indebted to the Chemistry Department of the University of Pittsburgh for permission to use this instrument.

An 0.01% solution of alloxan hydrate (in 5% trichloroacetic acid) kept at room temperature for many days showed no appreciable loss in chromogenic power in the pyrrole test; this may be contrasted with the change (6) shown by dehydroascorbic acid under the same conditions.

### *A Modification of Wöhler and Liebig's "Purple" Test for Alloxan*

Alloxan hydrate is readily reduced by a variety of reducing agents including ascorbic acid (9) and cysteine (10). In 1838, Wöhler and Liebig (11) observed that if barium hydroxide is added to the reduction product, a purple color or purple precipitate is formed. There has been a great deal of controversy as to whether the purple salt is barium alloxantinate or barium dialurate. Since the evidence in favor of one formulation as against the other is not particularly convincing we have temporarily referred to the test as the "purple" test.

We have now devised conditions under which, using as little as 1 cc. of 0.01% alloxan hydrate solution, and employing cysteine hydrochloride as the reducing agent, this purple color is readily detectable by the naked eye.

*Method of Performing the "Purple" Test on Pure Alloxan Monohydrate.* A solution (e.g., 0.01%) of alloxan hydrate in 5% aqueous trichloroacetic acid is prepared. A portion (1 cc.) of this solution is placed in a test tube, cysteine hydrochloride (about 15 mg.) is added and the suspension gently agitated until the hydrochloride has just dissolved. Cold, saturated (about 0.4 N) barium hydroxide solution is now added dropwise, but fairly rapidly, from a burette with gentle agitation of the mixture. As soon as the solution becomes alkaline a purple color develops. Addition of a few drops of barium hydroxide solution in excess gives a purple precipitate; a larger excess destroys the color. The color is more readily seen if the tube is held over a white surface illuminated by a fluorescent daylight lamp.

*Notes on the "Purple" Test.* It is advisable to perform a preliminary titration of a solution of 15 mg. of cysteine hydrochloride (in 1 cc. of 5% trichloroacetic acid) against the barium hydroxide solution, using phenolphthalein as indicator.

Cysteine hydrochloride is preferred to ascorbic acid as the reducing agent as excess of the latter gives, with excess barium hydroxide, a yellow color which tends to obscure the purple color given by very dilute solutions of alloxan. Although alloxan can also react with the amino group of cysteine, as with other amino acids, to give a murexide-type color, Lieben and Edel (12) have shown that reduction by its thiol group is the main reaction. That the color is not that of a purpurate is borne out by the fact that ascorbic acid may also be employed as the reductant. However, even were the purplish color of the murexide type it would still constitute a test for alloxan.

An 0.01% solution of ninhydrin (in 5% trichloroacetic acid) gives a red color which is discharged by excess barium hydroxide solution.

The "purple" test is of little value for materials containing much inorganic phosphate.

## EXPERIMENTAL

### *Preparation of Extracts*

(a) *Solids.* The fresh material was weighed and cut into small pieces (approximately 1 cm. cubes or smaller). It was now treated with one volume of 10% aqueous trichloroacetic acid in the Waring Blender, or macerated with sand in a mortar, and the mixture filtered through a fluted filter; the filtrate was vigorously shaken with charcoal, again filtered, and the filtrate tested.

(b) *Fluids.* One volume of 10% trichloroacetic acid was added to 1 volume of the fluid, the mixture filtered, and the filtrate treated as above.

*Application of the Pyrrole and "Purple" Tests to Extracts of Animal Materials.* Although treatment with charcoal will convert to alloxan any alloxantin or dialuric acid present, it is advisable to perform this treatment. With trichloroacetic acid extracts of liver, for example, it usually removes or greatly diminishes opalescence and completely eliminates the yellow color. In certain cases the original trichloroacetic acid extracts of liver were devoid of opalescence. The original trichloroacetic acid filtrates from blood and certain other materials are clear and colorless, but, to render the results comparable, these too were shaken with charcoal.

Extracts of certain animal materials responded to both tests: (see Table I).

We are inclined to believe that formation of the purple color is associated with the presence of alloxan (or its reduction products) for the following reason: the blood of normal untreated rabbits does not respond to the "purple" test but the blood of treated rabbits (which have received alloxan in the alimentary canal according to the technique of Ruben and Yardumian (13)) gives a positive "purple" test at 5 minutes and at 2 hours after commencing administration. Furthermore, the urine<sup>8</sup> of cats which had been fed alloxan responded to the "purple" test.

The first extract of human blood clot (accumulated from serological tests) gave a bright yellow color (resembling that produced by glutathione and cysteine) in the pyrrole test; the treated residue was reextracted with 10% acid as before and this second extract responded to both tests. Normal human blood plasma did not respond to either test, in agreement with Archibald's (4) results.

All samples of fresh liver tested (except those of two heart-death cases and of certain fish (1)) responded to the "purple" test. Extracts of the livers of fowl and duck gave relatively strong color tests, that of the duck being the strongest of any material so far tested. Trichloroacetic acid extracts of the fresh livers giving this test could be kept in the refrigerator for many days and still give the test.

## DISCUSSION

The two tests have been applied to certain deproteinized animal fluids and to aqueous trichloroacetic acid extracts of a variety of animal tissues. The results obtained suggest that alloxan or its reduction products may normally be present in a number of animal tissues.

<sup>8</sup> Collected from the 12th to the 24th hour after ingestion of alloxan.

TABLE I

*Results of Color Tests on Trichloroacetic Acid Extracts of Fresh Tissues and Fluids*

Material	Pyrrole Test (5-minute)	"Purple" Test
beef, liver	4+	1+
beef, spleen	3+	1+
calf, brain	4+	1+
calf, liver	4+	1+
calf, thymus	4+	1+
domestic fowl, liver	4+ to 5+	1+
duck, liver	6+	2+
guinea pig, liver	4+	1+
lamb, liver	2+	1+
pig, liver	4+	1+
rabbit, liver	4+	1+
rat, liver	2+	1+ to 2+
<i>Human, Autopsy</i>		
liver (premature twin)	3+	1+
serum from abdominal cavity (7-month, premature)	2+	1+
brain (7-month, premature)	5+	1+
liver (of a 4-day old)	3+ to 4+	1+ to 2+
liver (bronzed diabetic, 53 years old)	2+	1+
pancreas (bronzed diabetic, 53 years old)	2+	1+
liver (diabetic coma, 32 years old)	2+	1+
<i>Human, Surgical</i>		
uterine muscle	2+	1+
ovaries	3+	1+
thyroid	4+	1+

Key: 1+ = faint; 3+ = medium; 5+ = strong.

The fact that Ruben and Yardumian (13) were able to cause diabetes in the rat by introducing alloxan into the alimentary canal demonstrates that the diabetogen is not rapidly "destroyed" *in vivo*, since, in order to reach the general circulation, the alloxan (or a diabetogenic derivative thereof) presumably must be absorbed from the alimentary canal and pass through the portal circulation.

Of particular interest is the finding that alloxan or its reduction products may be present in the livers of normal animals. It was shown long ago (14) that an enzyme system which decomposes uric acid in the presence of oxygen, and synthesizes uric acid from dialuric acid plus urea in absence of oxygen, is present in dog and calf liver. It is further known that, allowing for variation in individual response,

alloxan is tolerated by normal animals, producing no observable adverse effect, when administered below certain critical concentrations. It exerts a rather general cytotoxic effect only when present in amounts above this threshold. Our results naturally suggest, as a tentative hypothesis, that overproduction of alloxan, or inability to destroy it, may result in its reaching the pancreas in sufficient concentration to destroy the beta cells in the islets of Langerhans.

The authors wish to express their gratitude to Dr. Leonard H. Cretcher for his interest in this work.

### SUMMARY

1. Two color tests of use in the qualitative detection of alloxan (or its reduction products) are described.
2. These two tests have been applied to trichloroacetic acid extracts of a variety of animal tissues and fluids. The results obtained suggest that alloxan (or its reduction products) may be a normal constituent of certain tissues.
3. These observations may be interpreted as indicating that overproduction of alloxan, or inability to destroy it, may result in a concentration of alloxan that is cytotoxic and eventuates in diabetes.

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# The Absence of Ricinoleic Acid from Phospholipids of Rats Fed Castor Oil

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Received June 25, 1945

## INTRODUCTION

By the use of tagged fatty acids—iodized acids (1), elaidic acid (2), deuteriumated acids (3), and spectroscopically active acids (4)—it has been demonstrated quite clearly that food fatty acids enter directly into the phospholipids of most, though not all, animal organs. The use of deuterium has also clearly shown that the fully saturated and monoethylenic acids are undergoing a continuous and rapid interconversion in the animal body (5), (6), (7). It might be assumed, therefore, from these two facts that the fatty acid composition of the tissue phospholipids is a simple function of the relative concentrations of the various fatty acids at the site of synthesis.

There is, however, a considerable body of evidence which indicates that the degree of freedom in the fatty acid make-up of the phospholipids is restricted within certain as yet ill-defined limits. For example, the relative proportion of fully saturated and unsaturated fatty acids appears to remain constant, the alteration in composition being due, as a rule, to a shift in the relative proportions of the various unsaturated acids (8), (9). Also, the phospholipids of beef liver contain, under normal conditions, a much higher proportion of arachidonic and other polyethylenic acids than is found in the liver glycerides (10), (11). So far as the authors know, a satisfactory interpretation of the mechanism underlying the fatty acid make-up of the phospholipids has not yet been proposed.

As a further contribution to the evidence bearing on the problem, the authors thought it worthwhile to determine whether or not ricinoleic acid, 12-hydroxy,  $\Delta^{9:10}$ -octadecenoic acid, would be utilized in the synthesis of phospholipids. Although castor oil acts as a cathartic for man and many animals, nevertheless considerable amounts of it

are absorbed (12), (13), (14). About 85% of the fatty acids of castor oil consists of ricinoleic acid. It is to be expected therefore that the intestinal mucosa especially, and probably other organs as well, would contain fairly large amounts of ricinoleic acid in animals that are absorbing large quantities of castor oil, unless the rate of metabolism of ricinoleic acid, by conversion or otherwise, is almost or quite as rapid as the rate of absorption.

### EXPERIMENTAL

*Diet:* Adult rats were fed for 25 to 40 days on a diet having the following composition: Castor oil 48.4%; casein 28.1%; dried yeast powder 14.4%; salt mixture (15) 6.3%; corn oil 1.4%; cod liver oil 1.4%. During the first few days the animals did not eat and a rapid loss of weight occurred. Aversion to the diet was soon overcome and, in most cases, the initial body weight restored. At no time was there any evidence of catharsis.

*Isolation of Fatty Acids:* The small intestine (after being flushed out with 0.9% NaCl solution), the liver and most of the muscle from the legs and back were ground thoroughly with crushed glass. The total lipids were extracted with boiling 95% alcohol. The phospholipid fatty acids were obtained as already described (16). The acetone-soluble fraction of the liver lipids was saponified and the non-saponifiable material removed.

Samples of the subcutaneous and perirenal fat were combined and saponified. The soaps were extracted with petroleum ether to remove non-saponifiable material.

From each rat, five samples of fatty acids were thus obtained—the phospholipid fatty acids of liver, small intestine and muscle, and the glyceride fatty acids of liver and fat depots. The fatty acids were esterified by refluxing for one hour with methyl alcohol containing 5% HCl. The alcohol was then evaporated, saturated  $K_2CO_3$  added, and the methyl esters extracted with ethyl ether. They were washed with 1%  $K_2CO_3$ , then with water, and finally dried and weighed.

*Determination of the Acetyl Number:* The ricinoleic acid content of the phospholipids and glycerides was determined by acetylation of the fatty acids.

The method of West, Hoagland and Curtis (17), somewhat modified, was employed. Aliquots of the methyl esters (50 to 100 mg. each) were transferred to dry glass-stoppered test tubes. One was kept for a blank titration; the other one (or two) was acetylated by heating for one hour with 0.0675 cc. of a mixture of acetic anhydride and pyridine (1:7), measured from a Rehberg-type burette. After being cooled, the stoppers and tubes were washed down with 1 cc. of 80% pyridine, and again heated for 20 min. to decompose the excess acetic anhydride. One or more blanks on the reagents were run at the same time.

To each tube, 5 cc. of *n*-butanol and 2 drops of 2% phenolphthalein were added and the acidity titrated with 0.02 *N* alcoholic NaOH.

As a check on the accuracy of the overall method, especially as saponification is said to dehydrate ricinoleic acid to some extent (18), varying amounts of castor oil were added to liver phospholipid and the fatty acids obtained by saponification

were analyzed for ricinoleic acid. The calculated acetyl numbers on the mixed esters were 7.4, 29 and 56; the values found were 7.8, 28 and 61. This agreement was regarded as satisfactory.

## RESULTS

Table I gives a summary of the data showing the *apparent* ricinoleic acid content of the fatty acids of control stock rats and of those fed castor oil. Since such hydroxy acids as occur in the lipids of animal tissues are normally restricted to the cerebrosides, and since these are a minor component of the lipids of liver, intestine and muscle, it was to be expected that the phospholipid fatty acids of these organs would possess a very low acetyl number, and that any increase in animals

TABLE I  
*Percentage Ricinoleic Acid in Fatty Acids of Rats Fed Castor Oil*

		Phospholipid fatty acids			Fatty acids of glycerides and cholesterol esters	
		Liver	Small intestine	Skeletal muscle	Depots	Liver
Control rats on stock ration	Average $\sigma$	1.7 (7) ±1.1	6.0 (4) ±4.4	4.0 (7) ±1.7	0.5 (7) ±0.5	5.6 (5) ±4.1
Rats fed 4-6 weeks on castor oil diet	Average $\sigma$	1.3 (9) ±0.6	4.9 (8) ±1.7	3.6 (8) ±2.9	6.8 (11) ±4.2	7.2 (8) ±2.4

The numbers in parentheses signify the number of analyses in each case.

absorbing castor oil could be safely interpreted as due to ricinoleic acid. It will be observed that the phospholipid fatty acids of these organs, and the glyceride and cholesterol ester fatty acids of the liver of rats on the stock diet showed small and rather variable acetyl values, equivalent to a few per cent of ricinoleic acid. The reason is not known. Storage of oils and fatty acids has been shown to cause an increase in acetyl number (19), presumably due to oxidation. It is thought that the acetyl absorption was probably due, in part at least, to traces of impurities in the fatty acids that escaped the precautions taken to eliminate them.

Comparison of the values for the control and oil-fed rats shows quite clearly that there is no indication that the feeding of castor oil

has led to the appearance of significant amounts of ricinoleic acid in the phospholipids of the small intestine, the liver and skeletal muscle, nor in the glycerides of the liver. On the other hand it is quite evident that ricinoleic acid is a component acid of the glycerides deposited in the depots, making up 7% of the total acids.

In the case of three rats, the feces were quantitatively collected during the period on diet. The fatty acids excreted amounted to 2.1, 2.2 and 3.6% of those ingested. In these same rats, the total body fat was determined. From the acetyl numbers it was calculated that only 1 to 2% of the ricinoleic acid that had been absorbed was deposited in the depots. It is evident therefore that ricinoleic acid is rapidly metabolized. Whether the apparently rapid and preferential metabolism involves complete combustion or elimination of the hydroxyl group is an interesting question. Present evidence does not offer an answer.

#### SUMMARY

(1) Rats were fed for several weeks on a diet containing 48.4% castor oil. No catharsis occurred. The fat excreted amounted to only about 2% of the intake.

(2) As there was no increase in the acetyl number over the control value, no evidence was found of the presence of ricinoleic acid in the phospholipids of the small intestine, liver and muscle, nor in the liver glycerides. However, ricinoleic acid made up about 7% of the fatty acids of the depot fat.

(3) Since only about 1 to 2% of the ricinoleic acid absorbed was deposited as fat, it is evident that this hydroxy acid is readily metabolized.

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# The pH Stability of Southern Bean Mosaic Virus

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Received June 25, 1945

## INTRODUCTION

In studies on the purification of southern bean mosaic virus (*Marmor laesiosaciens* Zaumeyer and Harter (1)), the preliminary results of which have already been published (2), it was desirable to know the pH range over which the virus would remain reasonably stable, since this would serve as a guide in the purification process and also tend to characterize the virus. A study of pH stability was therefore made. In three experiments the virus was exposed to different hydrogen ion concentrations for various periods of time and at different temperatures. This paper describes the experimental procedures employed and presents the results obtained.

## EXPERIMENTAL

A buffer was prepared at a concentration 0.05 *M* with respect to  $H_2BO_4$ ,  $KH_2PO_4$  and  $C_6H_4(COO)_2HK$ . Aliquots of the buffer were then adjusted to pH values between about 1 and 11, at intervals of approximately 1 pH unit, by the addition of NaOH or HCl. For each experiment, virus samples were brought to various pH's by adding 1 cc. portions to 10 cc. amounts of the different adjusted buffers. The pH values of the mixtures were determined immediately afterwards by means of a glass electrode, and again just before the virus samples were tested for activity. There was little or no change in pH of the samples during the period they were held under observation. Except in two instances, in which it was desired to test the effect of pH of the inoculum on the number of lesions produced, 1 cc. portions of each sample were brought to pH 7 by the addition of 9 volumes of 0.1 *M* neutral potassium phosphate buffer immediately before testing for infectivity.

The activity of each sample was determined by rubbing the test solutions on the upper surfaces of the primary leaves of from 9 to 15 Early Golden Cluster bean plants (*Phaseolus vulgaris* L.) in the 2-leaf stage. Lesions were counted 5 days later. This method of determining virus activity permits a fairly accurate comparison of lesions produced by different samples of virus on the same lot of bean plants provided

all inoculations are carried out at approximately the same time. It does not allow, however, an accurate comparison of lesion counts made on different days with different sets of bean plants. For this reason, the tables that follow give not only the actual lesion counts, but also the percentage activity based on the maximum lesion count for each particular test. The experiments were carried out in the following manner.

*Experiment 1.* Juice extracted from Bountiful bean plants that had been inoculated with southern bean mosaic virus 3 weeks previously was allowed to stand overnight at room temperature and was then centrifuged in an angle centrifuge for 20 minutes at 3000 r.p.m. This treatment removed much of the normal proteins and green colored material and yielded a clear, brown liquid. Aliquots of the liquid were buffered at different pH values and their activity tested immediately and after standing at a temperature of 3°C. for various periods of time.

*Experiment 2.* A virus preparation purified by chemical means, as described elsewhere (2), and containing 5.9 mg./cc. of protein was used in the second experiment. To 1 cc. aliquots of purified virus were added 9 cc. aliquots of the composite buffers at different pH values. The samples were held at a temperature of about 27°C. and tested for activity after various periods of time.

*Experiment 3.* Virus samples like those used in Experiment 2 were held at a temperature of about 3°C. By the 37th day it had become apparent that inactivation was occurring slowly over a wide pH range. The samples were then removed to a temperature of about 23°C. for 9 days when they were again tested for activity.

TABLE I

*Data Showing the Effect of pH on the Activity of Virus in Clarified Juice of Diseased Bean Plants Held at a Temperature of 3°C.*

pH	Length of time exposed to various H-ion concentrations							
	0 days*		1 day		7 days		28 days	
	Lesions (24 leaves)	per cent	Lesions (24 leaves)	per cent	Lesions (30 leaves)	per cent	Lesions (30 leaves)	per cent
1.4	0	0.0	0	0.0	0	0.0	17	1.0
2.6	15	0.5	111	1.9	90	2.4	3	0.2
3.3	18	0.6	841	14.7	1484	39.9	50	2.8
4.1	86	2.6	1880	32.9	3015	81.0	1260	71.1
5.2	686	21.1	3782	66.2	3721	100.0	1770	100.0
6.1	1613	49.5	3367	59.0	2832	76.0	1290	72.8
6.9	3257	100.0	5708	100.0	2520	67.6	535	30.2
7.9	3217	98.7	2877	50.3	2677	71.8	455	25.7
8.8	2581	79.4	1570	27.5	2289	61.5	78	4.4
9.6	30	0.9	21	0.4	7	0.2	1	0.1
10.5	0	0.0	3	0.1	0	0.0	—	—

\* Samples not neutralized before testing for activity.

## RESULTS

The data from the three experiments are summarized in Tables I, II and III. To determine the effect of pH on infectivity of the virus, in Experiments 1 and 3, samples were tested immediately after being

TABLE II

*Data Showing the Effect of pH on the Activity of Purified Virus Held at a Temperature of 27°C.*

pH	Length of time exposed to various H-ion concentrations					
	1 hour		1 day		7 days	
	Lesions (30 leaves)	per cent	Lesions (30 leaves)	per cent	Lesions (30 leaves)	per cent
1.2	0	0.0	2	0.0	—	—
2.3	1607	35.5	6	0.1	3	0.1
3.0	2423	53.6	251	5.2	2	0.1
3.9	2150	47.5	2485	51.5	1	0.0
5.0	2936	64.8	3196	66.1	2811	83.4
5.9	3388	74.9	4824	100.0	3371	100.0
6.7	4521	100.0	4331	89.6	3307	98.1
7.7	2723	60.2	1061	22.0	2	0.1
8.3	885	19.5	146	3.0	0	0.0
8.7	6	0.1	17	0.4	0	0.0
9.1	0	0.0	9	0.2	—	—

adjusted to the various pH values and without first being neutralized. In doing this, they were again diluted with 9 volumes of the buffers at the different pH values. The data show that the pH of maximum infectivity is between 6.2 and 7.9. It may be seen from the tables that the pH of maximum stability, on the other hand, is between 5.2 and 6.9.

The experimental results show that the pH stability of the virus is markedly dependent upon temperature. Activity decreased more rapidly and over a wider pH range at 27°C. (Table II) than at 3°C. (Tables I and III). In Table II it can be seen that activity decreased rapidly at 27°C. at pH 3.9 and below and at pH 7.7 and above. After 7 days at these pH ranges activity was almost completely lost. On the other hand, there seems to have been relatively little loss of activity between pH 5.0 and pH 6.7 during the 7 days the virus was held at

27°C. At 3°C. virus in clarified juice was rapidly inactivated below pH 3.3 and above pH 8.8 but it remained reasonably stable over the range pH 4.1 to pH 7.9 for 28 days (Table I). Comparable results were obtained with purified virus at the same temperature (Table III). There was rapid inactivation below pH 3.2 and above pH 8.6 but relatively little inactivation at or between these values for a period of 37 days. However, when the virus was removed to a temperature of 23°C., inactivation was rapid below pH 5.2 and above pH 6.9.

TABLE III

*Data Showing the Effect of pH on the Activity of Purified Virus Held at a Temperature of 3°C. for 37 Days and Then at 23°C. for 9 More Days*

pH	Length of time exposed to various H-ion concentrations									
	0 days*		4 days		10 days		37 days		46 days†	
	Lesions (50 leaves)	per cent	Lesions (50 leaves)	per cent	Lesions (50 leaves)	per cent	Lesions (50 leaves)	per cent	Lesions (50 leaves)	per cent
1.3	0	0.0	0	0.0	0	0.0	—	—	—	—
2.4	14	0.6	4	0.1	0	0.0	0	0.0	0	0.0
3.2	68	2.7	3183	70.2	2372	34.1	909	17.2	0	0.0
4.0	459	18.3	3640	80.2	6286	90.3	4120	78.1	124	7.8
5.2	1564	62.1	3923	86.5	6957	100.0	4632	87.6	377	23.6
6.2	1585	63.0	4442	97.6	4580	65.7	5076	96.3	1593	100.0
6.9	2518	100.0	3394	74.7	5173	74.3	5263	100.0	381	23.9
8.0	376	15.0	4541	100.0	5017	72.0	4478	85.0	75	4.7
8.6	74	2.9	840	18.5	1340	19.2	690	13.1	0	0.0
9.3	3	0.1	5	0.1	0	0.0	0	0.0	0	0.0
9.7	1	0.0	2	0.0	0	0.0	—	—	—	—

\* Samples not neutralized before testing for activity.

† 37 days at 3°C. and 9 days at 23°C.

Comparison of the data of Tables I and III, which represent results obtained with virus held at 3°C., indicate that there was little difference in the effect of pH on virus in partially clarified plant juice and in highly purified preparations. The minor differences observed can probably be ascribed to chance. Since the clarification process served to remove large particles and aggregated colloidal material, it is possible that somewhat different results would have been obtained with untreated infectious juice.

### DISCUSSION

The pH stability of a virus is obviously a function not only of the H-ion concentration of the solution but also of temperature. No definite statements can be made about the pH stability of southern bean mosaic virus without stating the temperature except that the pH of maximum stability, between pH 5 and pH 7, proved to be the same for two different temperatures. The evidence indicates that the pH of maximum stability does not coincide with the pH of maximum infectivity; the latter appears to be in a somewhat more alkaline range, pH 6.2 to pH 7.9. This finding is similar to that for other plant viruses. For example, it has been shown that while tobacco mosaic virus is relatively stable over a wide pH range, pH 2 to pH 8 (3, 4, 5), it is most infectious at pH 8 or pH 8.5 (3, 4). The virus of tomato spotted wilt is most stable between pH 6 and pH 9 (5, 6) but is apparently most active in more alkaline solutions up to pH 9.2 (5, 6). Tobacco necrosis virus in clarified plant juice was found most stable at a pH between 4 and 5 but most active in highly alkaline solutions up to about pH 10 (7). Potato mottle virus (potato latent mosaic virus) is essentially equally infectious at H-ion concentrations from pH 5 to pH 10 and its stability range for a 12-hour period is from pH 4 to pH 10 (8). Tobacco ringspot virus is stable only between pH 6 and pH 9 but is most infectious at pH 9.5 to pH 10 (9).

It has been pointed out by Stanley (9) that the influence of pH on the infectivity of a tobacco mosaic virus solution is dependent upon the host plant. In his experience, the susceptibility of *Nicotiana glutinosa* L. was not affected so markedly by changes in H-ion concentration as that of Early Golden Cluster bean plants. Nevertheless, his data appear to indicate that susceptibility of *N. glutinosa* did vary with pH and that the pH of maximum stability for both host plants is on the alkaline side of the pH stability range, i.e., between pH 8 and 9.

The effect of pH of the inoculum on susceptibility of the host may have a complex basis as suggested by Best and Samuel (5). It may depend, as they suggest, upon the effect on the protoplasm at the point of entry of the virus. The pH of the inoculum may determine the ability of the virus to enter living cells, or the ability of injured cells to recover once they have been entered, or by influencing the surface tension it may determine the degree of spreading of the solution

over the surface of the leaf. Whatever the nature of the effect, it is apparent that the pH of maximum infectivity and the pH of maximum stability are independent and do not, therefore, necessarily coincide in the case of any given virus-host relationship.

It is of interest to compare the pH stability range of southern bean mosaic virus with that of some other plant viruses. This has been

TABLE IV  
*Comparative pH Stability Ranges of Several Plant Viruses*

Virus	pH stability range	Time	Temperature	Literature cited
Southern bean mosaic	5.0- 6.7	7 days	27°C.	
Southern bean mosaic	4.0- 8.0	37 days	3°C.	
Tomato bushy stunt	1.5- 9.5	2 hrs.	18°C.	(11)
Tobacco necrosis	3.1- 9.7	24 hrs.	ca. 23°C.	(7)
Tobacco mosaic	1.5- 9.2	192 hrs.	20°C.	(3)
Tobacco mosaic	4.0-10.0	1 year	-14°C.	(3)
Tomato spotted wilt	6.1- 9.4	5 hrs.	0°C.	(5)
Cucumber mosaic	5.0- 7.0	1 hr.	20°C.	(3)
Potato mottle (= latent mosaic)	4.0-10.0	12 hrs.	0°C.	(8)
Tobacco ringspot	4.0- 9.5	2 days	4°C.	(9)
Tobacco ringspot	6.0- 9.0	5 days	4°C.	(9)
Alfalfa mosaic	5.0- 8.0	20 hrs.	4°C.	(10)
Alfalfa mosaic	5.5- 7.7	120 hrs.	4°C.	(10)

done in Table IV. An exact comparison is difficult because of the differences in time and temperatures involved in the various experimental results. It is evident, however, that the stability range of southern bean mosaic virus is considerably narrower than that of tobacco mosaic virus. It is probably also somewhat narrower than that of tobacco necrosis and tomato bushy stunt viruses, though reliable data for the latter virus are meager. Southern bean mosaic

virus appears to be less stable in highly alkaline solutions than tomato spotted wilt, potato mottle and tobacco ringspot viruses, but more stable in acidic solutions. It apparently has a wider pH stability range than either cucumber mosaic or alfalfa mosaic virus.

From the data that were obtained, it is apparent that southern bean mosaic virus can be handled with the least danger of inactivation if it is kept cool and at H-ion concentrations between pH 4 and pH 8.

### SUMMARY

The pH stability range of southern bean mosaic virus (*Marmor laesiofaciens* Zaumeyer and Harter) was determined for clarified juice samples at 3°C. and for purified virus at 3°C. and at 27°C. Little or no difference was found for the stability of the virus in clarified juice and in purified preparations but the pH stability range was much narrower at 27°C. than at 3°C. The virus was relatively stable at 27°C. for 7 days at pH 5.0 to pH 6.7, and at 3°C. for 37 days at pH 4.0 to pH 8.0. The pH of maximum stability (pH 5.2 to pH 6.9) did not coincide with the pH of maximum infectivity; the latter was in a somewhat more alkaline range, between pH 6.2 and pH 7.9.

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# Utilization of Metabolizable Energy by Growing Chicks

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Received June 20, 1945

## INTRODUCTION

In measuring the energy values of various feeds by experiments on growing chicks (1, 3, 4, 6) or growing rats (5), the productive energy was considered equal to the gains of energy in protein and fat made by the growing animal from the ration eaten in excess of that used for maintenance. This is represented by the equation (1)  $PF - WMD = G$ , in which  $P$  is the productive energy in calories per gram of the ration,  $F$  is the weight in grams of the ration eaten,  $W$  is the average live weight in grams of the animal for the period of the experiment,  $M$  is the calories of energy used for maintenance per gram of body weight,  $D$  is the number of days on experiment, and  $G$  is the number of calories gained by the animal. The average weight by weeks during the period of the experiment was found to give more consistent results with chickens than the surface area (1, 6).

## EXPERIMENTAL

The method of procedure involves the analysis of representative chickens at the beginning of each test, the individual feeding of the other chickens in battery brooders, usually for 3 weeks, and analyses of the chickens at the end of the experiments, together with digestion experiments on the rations used (1, 3, 4, 6).

The total energy content of the chickens or rats was calculated from the value of 5.66 calories per gram of protein ( $N \times 6.25$ ) and 9.35 calories per gram of fat, which was found by Fraps and Carlyle (1) to give the same results as combustion of chickens in a bomb calorimeter. The total energy stored was used in the calculation of the productive energy of the feeds and maintenance requirements of the animals in the work already reported.

The metabolizable energy values per gram of the mixtures were ascertained in a number of digestion experiments with young growing chickens by subtracting the caloric value of the excrement from that of the feed mixture eaten and then dividing the difference by the grams of the feed mixture eaten (2). The values so secured were much higher than the values calculated from the digestible nutrients by use of the factors of 4.2 calories per gram of nitrogen-free extract and crude fiber, 9.47 for ether extract and 4.0 for protein. The discrepancy was due to the retention of protein, averaging 56.6% of the digested protein. To secure correct values for the metabolizable energy, corrections were made for the non-metabolizable energy retained in the flesh gained by the chickens. Otherwise, the values secured for metabolizable energy would vary according to the quantity of protein retained. In these particular experiments, the metabolizable energy averaged 5.13 calories per gram of digestible protein when correction was not made for the protein stored, but when correction was made for the non-metabolizable energy in the protein stored, the value was 4.4 calories per gram (2). This raised the question whether the total energy in the protein stored should be used in calculating the productive energy of the ration, or whether the metabolizable energy in the protein stored should be used. If the metabolizable energy of the ration cannot be correctly calculated unless correction is made for the non-metabolizable energy in the protein stored in the growing chicken, then perhaps the percentage of the metabolizable energy used for production of flesh is also incorrect unless a similar adjustment is made. In order to throw light on this subject, representative data from the previous 192 tests have been recalculated on the basis of the metabolizable energy of protein stored instead of the total energy stored by the growing chicks. The only change in the data is from total energy to metabolizable energy of the protein stored by the chickens. The data and the methods of calculation previously used have been presented in full.

## DISCUSSION

### *Relation of total energy to metabolizable energy content of chickens.*

The metabolizable energy in the chickens is calculated from the analyses previously reported by using 4.4 calories per gram for metabolizable energy of protein instead of 5.66 for total energy. The protein content of chickens does not vary to a great

extent but decreases slightly as the fat content increases. The metabolizable energy gained with some chickens of different fat content, as compared with total energy gained, is shown in Table I. The differences in calories per 100 grams found by the two methods of calculation are nearly constant, ranging from 25 calories with chickens containing 12% fat to 29 calories with those containing 2.62% fat. On a percentage basis, the differences are greater. The metabolizable energy ranges from 81.3% of the total energy with chickens containing 2.62% fat to 88.8% with chickens containing 12% fat.

*Comparison of productive energy of the rations.*

The calories of productive energy of representative rations were calculated from data secured by feeding two groups of chickens at the

TABLE I  
*Relation Between Total Energy and Metabolizable Energy Stored by Chickens as Affected by Fat Content*

Fat in chickens per cent	Protein in chickens per cent	Total energy calories per 100 grams	Metabolizable energy calories per 100 grams	Metabolizable energy in percentage of total energy
2.62	22.7	147.3	118.7	81.3
2.97	22.9	157.6	128.9	81.7
7.38	21.5	183.7	156.6	85.8
7.55	21.0	189.6	163.1	86.0
9.31	19.6	197.9	173.2	87.5
12.00	20.0	225.2	200.0	88.8

same time on the same rations, one group receiving full feed and the other group only about half this quantity (1). By use of the data for the two groups and the equation given above, the productive energy of the ration was previously calculated in terms of total energy stored. For the purpose of the work here presented, the productive energy was recalculated in terms of the metabolizable energy stored (1). The results are compared in Table II.

The productive energy of the ration on the basis of the metabolizable energy stored by the chickens was 92.0 to 93.3% of that calculated on the basis of the total energy stored, with an average of 92.7% for 5 tests, i.e., 7.3% lower. The same proportion would apply to the total ration, its effective organic constituents, its effective digestible nutrients and its metabolizable energy.

*Energy used for maintenance.*

The productive energy used for maintenance was calculated on the total energy basis from the data secured with several corn meal rations, using the equation given above and the value of 2.78 calories of productive energy per gram of effective digestible nutrients (3, 4, 6). For the metabolizable energy basis, the productive energy used for maintenance was calculated from the same data for some representative experiments (3), with use of 92.7% of 2.78, or 2.58, calories per gram of effective digestible nutrients. The results are given in Table III.

TABLE II  
*Productive Energy Calculated from Total and from Metabolizable  
 Energy Stored by Chickens*

	Exp. 55	Exp. 56	Exp. 60	Exp. 57	Exp. 59
Length of experiment, days	21	21	21	42	42
Full feed					
Weight by period, g.	121.8	114.0	99.8	212.9	242.0
Feed eaten, g.	361.5	360.9	316.8	1120.9	1291.0
Total energy gained, cal.	351.9	309.7	252.2	808.2	913.7
Metabolizable energy gained, cal.	312.3	270.4	195.5	701.7	793.3
Limited feed					
Weight by periods, g.	75.9	71.7	69.9	100.4	112.1
Feed eaten, g.	164.7	165.7	171.0	430.8	488.7
Total energy gained, cal.	97.0	83.3	72.3	163.7	208.9
Metabolizable energy gained, cal.	80.5	66.1	58.3	129.0	169.0
Productive energy per gram of food					
Total energy basis (T) cal.	2.019	1.819	1.679	1.958	1.967
Metabolizable energy basis (M)	1.884	1.696	1.545	1.810	1.816
Per cent M of T (average 92.7)	93.3	93.2	92.0	92.7	92.6

There is little difference in the figures for the energy used for maintenance per day per 100 grams secured by the two methods of calculation. In the equation  $WMD = PF - G$ , the differences due to the lower energy value of the ration on the metabolizable energy basis in these experiments are nearly neutralized by the lower metabolizable energy content of the chickens. Since the rations have about 7% lower productive energy value when calculated on the metabolizable energy basis than on the total energy basis, while the calories used for maintenance per day per 100 grams are nearly the same, the quantity of the

TABLE III

*Energy Used for Maintenance Calculated from Total Energy Gained and from Metabolizable Energy Gained by Chickens*  
Time of experiment—3 wks.

	Exp. 9	Exp. 11	Exp. 12
Corn meal ration			
Weights by period, g.	131.5	140.4	124.0
Feed eaten, g.	322.8	315.9	280.0
Total energy basis			
Productive energy of ration, cal./g.	1.979	1.876	1.980
Productive energy of feed eaten, cal.	638.8	592.6	631.7
Gain of energy, cal.	291.3	286.1	251.3
Used for maintenance, cal., total	347.5	306.5	280.4
Used for maintenance, per day and per 100 g., cal.	12.59	10.40	10.47
Used for maintenance, g. ration per day and per 100 g. (T)	6.36	5.54	5.67
Metabolizable energy basis			
Productive energy of ration, cal./g.	1.835	1.739	1.760
Productive energy of feed eaten, cal.	592.3	549.4	492.8
Gain of energy, cal.	247.6	245.3	214.0
Used for maintenance, cal.	344.7	304.1	278.8
Used for maintenance, per day and per 100 g., cal.	12.48	10.30	10.70
Used for maintenance, g. ration per day and per 100 g. (M)	6.80	5.92	6.08
Per cent M of T of ration used	107	107	107

ration required for maintenance is about 7% more when calculated on the metabolizable energy basis than when calculated on the total energy basis.

#### *Relative value of feeds.*

The productive energy values of various feeds were compared by the use of the data secured by feeding usually four groups of chickens, one on a corn meal ration and the other three on rations in which corn meal or corn meal and casein were replaced with feeds to be compared. Analyses of the chickens provided their gains of energy.

The productive energy of the feeds calculated on a metabolizable energy basis would be practically 92.7% of that calculated on a total energy basis if the fat contents of the chickens were the same on all

four groups compared in any particular experiment. Table I shows that the ratio of metabolizable to total energy in the chickens depends on their fat content. In some of the experiments, the fat contents of the chickens produced on the 4 rations were quite different from one another. In order to ascertain the effect of such differences, experiments were selected for recalculation in which the chickens on the experimental rations developed wide differences in fat content from those on the corn meal ration. The results of these calculations (3) are given in Table IV.

TABLE IV

*Relative Value of Feeds in Productive Energy Determined on the Metabolizable Energy Basis as Compared with That on Total Energy Basis*

Time of experiment — 3 wks.

	Corn meal	Corn bran	Wheat gray shorts	Wheat bran	Corn meal	Casein	Starch	Cot- tonseed oil	Corn meal	Casein	Starch	Cot- tonseed oil
Experiment Number	9	9	9	9	11	11	11	11	12	12	12	12
Per cent fat in chicken	7.38	3.13	2.97	2.62	7.38	2.62	9.58	9.87	7.55	2.84	9.31	12.00
Productive energy of feed, cal./g., total energy basis												
T	2.480	1.212	1.698	0.936	2.478	2.042	2.193	3.513	2.430	2.139	1.941	4.409
Productive energy of feed, cal./g., metabolizable energy basis M												
M	2.298	1.088	1.530	0.842	2.297	1.750	2.055	3.420	2.253	1.932	1.848	4.293
Per cent M of T	92.7	89.5	90.1	90.0	92.7	85.7	93.7	97.4	92.7	90.3	95.2	97.4

The data show that when the fat content of the chickens on the test ration is about 3%, as compared with about 7% in the chickens on the corn meal ration, the productive energy of the digestible constituents relative to corn meal is lowered about 3%. When the fat content is 9 to 12% in the test rations as compared with 7% in the corn meal ration, the relative value is increased about 3%. These changes are practically within the limit of error.

#### SUMMARY

The metabolizable energy stored by young growing chickens depends to some extent on the fat content and is 81.3% to 88.8% of the total energy of chickens containing 2.6 to 12.0% fat respectively. The productive energy of a corn meal ration calculated from the metabolizable energy stored averages 92.7% of that calculated from the total energy stored. The productive energy used for maintenance is practi-

cally the same on the metabolizable energy basis as on the total energy basis in the experiments studied, but since the values differ by about 7%, the quantity of feed calculated to furnish the energy for maintenance is about 7% higher on the metabolizable energy basis than on the total energy basis. The total energy basis may not be a correct method to calculate the metabolizable energy stored by animals from feeds, since part of the energy stored is from non-metabolizable energy and corrections must be made for the non-metabolizable energy in ascertaining the metabolizable energy of feeds.

Whether the total energy stored by the animal or the metabolizable energy stored should be used in determining the productive energy of feeds, remains to be decided, but the productive energy heretofore reported has been calculated from the total energy stored.

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# Vitamin and Protein Content of Residues from the Production of Penicillin by Submerged Fermentation

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Received July 5, 1945

## INTRODUCTION

The recognition of the high nutritive value of ethanol and acetone-butanol fermentation residues and their recovery as dried feed supplements have aided greatly in the solution of a serious waste disposal problem. Moreover, the recovery of these by-products has provided an additional source of protein and vitamin concentrates for animal and poultry feeds, and a further credit to the manufacturer. The production figures and nutritive value of alcohol fermentation by-products have been reviewed recently by Bauernfeind *et al.* (1, 2, 3). By-products rich in riboflavin which are produced during the acetone-butanol fermentation have been described by Miner (4) and by Arzberger (5).

The rapidly increasing production of penicillin by submerged culture methods has once more created a plant waste disposal problem. As part of a survey of vitamin synthesis by fungi being carried on at this Laboratory, vitamin production by *Penicillium chrysogenum* cultivated in corn steep liquor-lactose-salts medium has been investigated. Vitamin determinations have been made on (a) the uninoculated medium, (b) the medium and mycelium during the course of fermentation, (c) the products resulting from the recovery of penicillin by the carbon method, and (d) the fermentation liquor and mycelium after evaporation and drum-drying. The results indicate that penicillin fermentation residues are comparable to alcohol fermentation by-products in their content of protein and several water soluble vitamins,

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and that they are markedly more potent than the latter in pantothenic acid content. Animal tests to establish the value of this fermentation residue as a feed ingredient are now in progress.

### METHODS

The medium employed was that described by Moyer and Coghill (6) for the production of penicillin by the surface culture technique and later modified for penicillin production by the submerged process.

The modified medium had the following composition:

Lactose	30 g.
Corn steep liquor	80 g. (approximately 50% solids)
NaNO <sub>3</sub>	3 g.
CaCO <sub>3</sub>	10 g.
Tap water to make	1000 ml.

The above constituents, with the exception of the calcium carbonate, were placed in 35 liter quantities in two 50-liter aluminum fermenters and were sterilized at 17 pounds steam pressure (per sq. in. gage) for 60 minutes. After cooling, sterile, dry calcium carbonate was added at the rate of 10 g. per liter of medium. The pH before inoculation was 4.8. Each fermenter was inoculated with one liter of a 48 hour culture of *Penicillium chrysogenum*, NRRL 1951.B25. The medium, maintained at a temperature of 24–25°C., was agitated continuously and aerated at the rate of 0.6 volume of air per volume of medium per minute.

Solids, nitrogen, pH, residual lactose, and vitamins were determined on samples of uninoculated medium and on culture filtrates and mycelium during the course of the fermentation. Mycelium was separated from the liquor by vacuum filtration and was dried overnight at 105°C. without attempting to wash it free of culture liquor. For analysis, the dried mycelium was finely ground. The culture liquors were assayed in the wet state following the pretreatment described below.

After 137 hours incubation the contents of the two fermenters were combined (after frequent sampling 18.8 liters remained in fermenter A and 16.2 liters in fermenter B) and were thoroughly mixed. Mycelium was separated by filtration through cloth and the filtrate (25.8 liters) was adjusted to pH 5.7 with sulfuric acid. The filtrate was concentrated under reduced pressure (approximately 25 mm.) to approximately 3 liters. The mycelium was then mixed with the concentrated liquor and the mixture dried on a laboratory drum-dryer employing steam at 110 lb. pressure in the drums.

Vitamins were determined by microbiological methods. Samples for riboflavin assay were extracted by autoclaving for 30 minutes at 15 pounds in the presence of 0.1 N HCl after which the method of Snell and Strong (7) was used. Pantothenic acid was determined by the method of Pennington *et al.* (8). Materials to be assayed were digested for 24 hours with clarase and papain. Results were corrected for the pantothenic acid content of the enzymes. Niacin was determined by the procedure described by Snell and Wright (9) after the samples were extracted by autoclaving as described above for riboflavin for 30 minutes in the presence of

1 N NaOH. For biotin and pyridoxin determination, materials were hydrolyzed with 5 N H<sub>2</sub>SO<sub>4</sub> in the autoclave for 30 minutes at 15 pounds after which biotin was assayed by the method of Wright and Skegg (10) and pyridoxin by the method of Atkin *et al.* (11). Nitrogen was determined by Kjeldahl procedure, and solids by drying the samples for 12 hours at 105 C. The method of Schmidt and Moyer (12) was employed for determining penicillin. Lactose was determined by a modification of the Shaffer-Hartmann method (13).

## RESULTS

### *Production and Distribution of Vitamins*

Fermentation data are presented in Table I. The utilization of nutrients was more rapid and complete in fermenter B than in fer-

TABLE I

*Penicillin Production by the Submerged Fermentation of Sheep Liquor-Lactose Medium with *P. chrysogenum*, NRRL 1951.B25*

	Fermentation data					
	Fermenter A		Fermenter B			
	Fermentation time, hrs.					
	0	96	137	0	96	137
Lactose, g./100 ml.	2.9	2.0	1.1	2.9	1.4	0.4
pH	4.8	6.6	7.1	4.8	7.2	7.3
Penicillin, Units/ml.	—	32.0	77.0	—	50.0	112.0
Total solids, g./100 ml.	6.9	6.0	5.3	7.5	5.4	4.0
Solids in filtrate g./100 ml.	—	4.7	3.0	—	3.3	2.0
Mycelium, g./100 ml.	—	1.3	2.3	—	2.1	2.0

menter A as evidenced by the lower residual sugar values, lower solids in the cell-free filtrate and the earlier appearance of an alkaline reaction. Penicillin production was also greater in fermenter B. The vitamin potencies, however, were quite similar in the two fermentations (Table II). The increase in pantothenic acid during fermentation is most pronounced although all vitamins were synthesized to some extent. The liquor rose from an initial pantothenate potency of 1.9 γ/ml. to 17 γ/ml. which, coupled with the level in the mold mycelium, gave an overall increase of approximately tenfold. Pyridoxin and biotin were approximately doubled in the cell-free liquors while

TABLE II

*Vitamin Synthesis During the Submerged Fermentation of Steep Liquor-Lactose Medium by *P. chrysogenum*, NRRL 1951.B25*

	Vitamin content									
	Fermenter A					Fermenter B				
	Liquor		Mycelium			Liquor		Mycelium		
	Fermentation time, hrs.									
	0 <sup>1</sup>	96	137	96	137	0 <sup>1</sup>	96	137	96	137
Riboflavin γ/ml.	0.4	0.8	0.2			0.4	0.5	0.6		
Niacin γ/g. dry substance	5.7	16.1	7.3	32.0	47.5	4.9	15.7	29.0	55.2	39.8
Pantothenic acid γ/ml.	6.5	8.9	7.0			6.6	5.0	6.7		
	93.9	188.0	232.0	35.2	212.2	88.0	164.0	333.0	180.2	201.0
Pyridoxin γ/ml.	1.8	11.9	17.9			2.0	12.8	16.1		
	26.4	250.0	582.0	22.8	107.7	26.8	382.0	805.0	100.2	140.7
Biotin γ/ml.	1.8	1.2	2.5			1.3	2.4	2.9		
	25.4	26.0	82.2	8.8	24.6	17.8	70.9	144.0	26.2	20.9
	0.01	0.026	0.018			0.010	0.019	0.017		
	0.142	0.555	0.608	0.290	1.005	0.138	0.563	0.826	0.448	0.570

<sup>1</sup> Uninoculated medium.

niacin and riboflavin values were only slightly above those in the basal medium. On the dry basis, however, the potency of all vitamins was raised because of the loss in solids in the form of volatile materials during fermentation. The mycelium, especially in fermenter A, showed an increase in potency between 95 and 136 hours, part of which may have been the result of absorption of vitamins from the medium during this period.

*Vitamins in Liquors Resulting from Penicillin Recovery*

Since several of the water soluble vitamins have been shown to be largely absorbed under certain conditions by activated carbon (14, 15, 16), the fate of the vitamins contained in the cell-free liquor was followed during the carbon method of penicillin recovery.

A sample of fermented liquor was filtered free of mycelium and the filtrate was adjusted to pH 5.8 with phosphoric acid. To the filtrate (1400 ml.) 1.5% activated carbon (Darco G-60) was added and the mixture agitated continuously for 15 minutes. The Darco was removed by filtration and eluted with amyl acetate and water (23%

amyl acetate by volume). The volume of eluate recovered was 80 ml. Results of vitamin assays conducted on both the filtrate and the eluate fractions are found in Table III.

With the exception of pantothenic acid, a major portion of the vitamins was adsorbed from the liquor with the penicillin. The liquor, after adsorption and removal of the Darco, contained only 16% of the riboflavin, 18% of the niacin, 4% of the pyridoxin, 33% of the biotin, and 76% of the pantothenic acid originally present. The Darco eluate, which was much higher in vitamin potency because of its smaller volume, contained only 3% of the riboflavin, 28% of the

TABLE III

*The Vitamin Content of Liquors Resulting from the Recovery of Penicillin by the Adsorption-Elution Method*

Fraction	Solids per cent	Vitamin content					Distribution Per cent of total in each fraction				
		Ribo- flavin γ/ml.	Niacin γ/ml.	Panto- thenic acid γ/ml.	Pyri- doxin γ/ml.	Biotin γ/ml.	Ribo- flavin	Niacin	Panto- thenic acid	Pyri- doxin	Biotin
Clarified, fermented liquor (1400 ml.)	2.48	0.5	7.0	18.0	2.8	0.018	100	100	100	100	100
Darco filtrate (1380 ml.)	2.00	0.08	1.3	13.9	0.12	0.006	15.8	18.3	76.0	4.2	32.9
Darco eluate (80 ml.)	4.24	0.30	34.5	47.1	12.1	0.120	3.4	28.2	14.9	24.7	38.1
Darco (by difference)							80.8	53.5	9.1	71.1	29.0

niacin, 15% of the pantothenic acid, 38% of the biotin, and 25% of the pyridoxin. The Darco, under these conditions, retained considerable quantities of all vitamins except pantothenic acid.

#### *Analysis of Dried Product*

As described previously, after 137 hours of incubation the liquors from the two fermenters were combined and dried. In Table IV, the composition of the dried product is compared with distillers' solubles produced as a by-product in the alcoholic fermentation of grains. Since it was not possible to carry out the recovery of penicillin from all the material, the analysis for the product prior to carbon treatment is given. The loss in vitamins from the liquor by the recovery procedure is calculated from the data in Table III.

In spite of the loss suffered in carbon treatment, the fermentation residue compares favorably with distillers' solubles in protein, niacin,

riboflavin and biotin, and is markedly more potent in pantothenic acid and pyridoxin. The yield of dried product in this experiment was 0.33 pound per gallon of mash. It has been estimated that approximately 0.25 pound of dried product could be derived per gallon of commercial medium.

### *Vitamin Synthesis by Other Strains of *P. chrysogenum**

Because the study of vitamin synthesis in submerged culture was carried out on only a single strain of *Penicillium*, it was considered of

TABLE IV  
*Chemical Composition of Penicillin Medium, Penicillin Residues, and an Alcohol Fermentation By-product from Corn*

Product	Protein (N × 6.25)	Ash per cent	Ribo- flavin γ/g.	Niacin γ/g.	Panto- acid γ/g.	Pyri- doxin γ/g.	Biotin γ/g.
Unfermented medium <sup>1</sup>							
Average of fermenters A and B	28.2		5.3	91.0	26.6	21.6	.142
Fermented medium com- posed of:							
a. Mycelium and un- treated culture filtrate	30.5	20.3	28.0	167.6	393.0	44.7	0.578
b. Mycelium and Darco-treated culture filtrate (calculated from Table III)							
Distillers' dried corn solubles <sup>2</sup>	27.0	8.0	25.6 15-20	128.1 140-160	340.0 29-36	38.4 8-10	0.425 2.0-2.4

<sup>1</sup> The analysis of penicillin products is given on a 5% moisture basis; distillers' solubles on a 10% moisture basis.

<sup>2</sup> From Bauernfeind *et al.* (1).

interest to compare vitamin synthesis, especially of pantothenic acid and pyridoxin by several strains of *Penicillium*. For this purpose nine strains were investigated. Cultures were grown on 175 ml. of steep liquor-lactose-salts medium (17) in 1 liter Erlenmeyer flasks which were shaken continuously for one week at 24-25°C. Penicillin production and vitamin synthesis are shown in Table V.

The maximum potency of penicillin produced by these cultures during a seven-day period varied from 29 units per ml. with strain P.S. 264 to 142 units per ml. with culture NRRL 1951.B11. While biotin and niacin syntheses varied only slightly between cultures, larger differences were noted in the contents of riboflavin, pyridoxin and pantothenic acid. Culture P.S. 264 which was a poor producer of penicillin gave relatively high yields of riboflavin and pyridoxin. Culture 1951.B11 which gave the highest penicillin potency gave near-

TABLE V  
*Vitamin Synthesis by Strains of Penicillin Varying in Their Ability to Produce Penicillin*

Strain <sup>1</sup>	Penicillin yield Units/ml. <sup>2</sup>	Total Solids per cent	Riboflavin γ/ml.	Pantothenic acid γ/ml.	Niacin γ/ml.	Pyridoxin γ/ml.	Biotin γ/ml.
1951.B25 (a)	110	3.84	.94	10.8	5.7	2.8	.027
1951.B25 (b)	90	4.50	.96	10.0	6.1	2.2	.028
1951.B11 (a)	142	3.53	1.07	11.7	5.6	2.1	.028
1951.B11 (b)	94	3.54	1.00	9.4	5.7	2.2	.026
P.S. 261 (a)	77	3.78	.78	9.15	6.0	2.2	.023
P.S. 263 (a)	104	3.75	.75	12.4	5.7	1.7	.024
P.S. 264 (a)	99	3.99	.87	14.3	5.7	2.3	.023
P.S. 264 (b)	29	3.25	1.36	10.7	6.1	3.2	.026
832 (Abbott)	67	3.90	.92	13.9	5.9	2.3	.024

<sup>1</sup> Strains (a) and (b) were different colony isolates from the respective parent strain.

<sup>2</sup> Highest penicillin potency in Units reached during the seven day incubation period.

average yields of all vitamins. Pantothenic acid production was approximately two-thirds that found in the vat fermenters. This may be the result of lower steep liquor concentration employed or to a difference in metabolism between cultures aerated by shaking and those aerated by the direct introduction of air.

#### ACKNOWLEDGMENT

The authors gratefully acknowledge the cooperation of various members of the Fermentation Division, Northern Regional Research Laboratory, in carrying out this investigation. Fermented liquors and some of the fermentation data were supplied by Drs. G. E. Ward and K. B. Raper. The adsorption and elution of penicillin from culture liquors were performed with the assistance of Dr. J. L. Wachtel

and Mr. Morris Friedkin, and liquors were assayed for penicillin by Mr. W. H. Schmidt.

### SUMMARY

Vitamin synthesis by *P. chrysogenum* cultivated under submerged conditions in steep liquor-lactose medium has been investigated. Pantothenic acid was increased approximately tenfold over that in the basal medium while the pyridoxin potency was doubled during the 137 hour fermentation period. The other B vitamins investigated, biotin, riboflavin and niacin, were increased only slightly.

With the exception of pantothenic acid, vitamins from the clarified fermented liquor were largely adsorbed on the Darco along with the penicillin. A single elution with aqueous amyl acetate was only partially effective in removing the adsorbed vitamins from the Darco.

The dried residue, composed of mold mycelium and residual liquors after penicillin removal, was comparable to alcohol fermentation by-products in the content of protein, niacin and riboflavin and much more potent in pantothenic acid and pyridoxin.

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# The Formation of Split Diphtheria Antitoxic Pseudoglobulin and its Combination with Pepsin

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Received July 12, 1945

## INTRODUCTION

When horse or beef pseudoglobulin is subjected to peptic or tryptic digestion in moderately acid medium hydrolysis does not immediately go to completion, but first produces a new pseudoglobulin whose molecular weight is only 60% that of the native substance. When the material digested is diphtheria antitoxic pseudoglobulin, the new protein has the same antitoxic properties as the original (1-5). The same proteolysis can occur spontaneously, without the help of an added enzyme, in antitoxic pseudoglobulin solutions which have been stored for a number of years (6). The smaller molecules thus produced are more resistant to denaturation than fresh pseudoglobulin and flocculate with toxin at a much faster rate. Their lower molecular weight is reflected in the smaller amount of antitoxin nitrogen that combines with toxin in optimal ratio, but the mode of the flocculation is not otherwise essentially different from that of fresh antitoxin (6). We have used the term "split" antitoxin to designate this new antitoxic pseudoglobulin of reduced molecular weight, whether it is produced artificially with the help of some proteolytic enzyme or spontaneously during storage.

Pepsin is known to be removed from solution by combining with insoluble proteins. This fact afforded Northrop an opportunity for an extensive investigation of the combination of pepsin with edestin crystals (7). That a similar study can be carried out with a very soluble protein is shown here in an investigation of the combination of pepsin with split diphtheria antitoxic pseudoglobulin. This combination is characterized by complete reversibility and by great stability, due to the marked resistance of split antitoxin to peptic digestion in

moderately acid medium. The present paper reports experiments on the formation of split antitoxin from native antitoxin, and a quantitative study of the combination of split antitoxin with pepsin.

### MATERIALS AND METHODS

*Pepsin.* Parke, Davis and Company 1:10,000.

*Diphtheria Antitoxin No. 992.* Diphtheria antitoxic pseudoglobulin concentrated about 18 months previously by the method current in this laboratory (8). The solution contained 175 mg. of protein, 9 mg. of NaCl, 3 mg. of cresol and 2500 Lf per ml. Molecular weight, determined osmotically by the author's method (9, 10), 164,000.

*Pepsin-treated Diphtheria Antitoxin No. 1013.* Diphtheria antitoxic pseudoglobulin, concentrated by the standard method, was treated with pepsin at pH 4.0, and purified by a method based on Pope's procedure (1, 11). The solution contained 175 mg. of protein, 9 ml. of NaCl, 3 mg. of cresol, and 3700 Lf per ml. Molecular weight 116,000. Referred to as "split" antitoxin. Both solutions were dialyzed against tap water before use.

Antitoxin No. 992 was 16.0% precipitable by toxin; antitoxin No. 1013, 16.7% precipitable. Both solutions may thus be said to have consisted approximately of 16% active antitoxin molecules, and 84% inactive molecules. In this laboratory, and quite generally in scientific publications, the term antitoxic serum is used to designate whole serum that possesses neutralizing activity against the specific toxin employed in its production, and the term antitoxin for the pseudoglobulin fraction separated from such a serum. Though only part of such pseudoglobulin preparations is actually antitoxic, there are reasons for believing (2) that the nonantitoxic part is probably not identical with normal pseudoglobulin.

*Protein Determinations.* Protein nitrogen was determined by micro-Kjeldahl. Native antitoxin was assumed to contain 14.3% nitrogen (12), crystalline pepsin 14.3% (13). Split antitoxin freshly dialyzed against distilled water contained 14.7% nitrogen.\*

Total protein was determined with the Zeiss dipping refractometer. Each scale division was taken as expressing 0.207% concentration (14), a factor found valid, within a small error, for native antitoxin, split antitoxin, and crystalline pepsin.

*Tyrosine-Tryptophan.* To 3 ml. of digestion mixture 10 ml. of 10% trichloroacetic acid were added and the precipitate filtered off. To 1 ml. of filtrate 4 ml. of 12%  $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$  were added followed by dropwise addition of 0.5 ml. of Folin's reagent diluted 1:3. The color was read between 30 and 60 minutes later in a photo-electric colorimeter. Appropriate blanks were made. For a standard, 30 mg. of tyrosine in 1 liter of 0.2 N HCl were used.

\* The difference in the nitrogen content between native and split antitoxin may be within the limits of experimental error. The carbohydrate content of both proteins is probably the same (4). The color per unit concentration developed by split antitoxin No. 1013 with Folin's reagent in the presence of 0.001%  $\text{CuSO}_4$ , was 1.16 times that given by native antitoxin No. 992.

*Analysis of Antitoxin-Pepsin Complex.* The precipitate was dissolved in 0.1 N HCl and incubated one hour at 37°C. This procedure hydrolyzes the antitoxin but not the pepsin. The composition of the compound was calculated from the difference between total nitrogen and nitrogen not precipitated by the addition of an equal volume of boiling 10% trichloroacetic acid.

*Antitoxin Titrations.* All titrations were made at 37°C. in 75 × 11 mm. tubes containing 25 Lf of toxin and increasing amounts of antitoxin in a total volume of 1.0 ml. of neutral buffer. Split antitoxin flocculates over a wider range of toxin:antitoxin ratios than does native antitoxin, and titrations are consequently less exact. The simplest and most satisfactory procedure consisted in increasing the antitoxin amounts serially by the factor 5/4, and noting not only the time at which the first tube flocculated, but also when flocculation occurred in the neighboring tubes. For example: if in tubes A, B, C, and D, in which the amounts of antitoxin increase serially by the factor 5/4, flocculation occurs after 40, 12, 8, and 13 minutes respectively, the optimal toxin:antitoxin ratio is in tube C. If the times are 40, 12, 13, and 50 minutes, the optimal ratio would be midway between B and C; if the times are 40, 12, 20, and 50 minutes, the optimal ratio would be between B and C, but nearer to B. The correctness of the method is shown by the fact that the plotting of the flocculation time against the logarithm of the toxin:antitoxin ratio yields a parabolic curve that is nearly symmetrical. The results are probably accurate within 5%. The presence of even large amounts of pepsin was without effect on the flocculation rate of antitoxin as long as the pH was near neutrality.

## EXPERIMENTAL

### *Formation of Split Antitoxin*

In the experiments reported, the progress of total hydrolysis was indicated by the amount of antitoxin left and of nonprotein nitrogen produced, and the progress of partial hydrolysis by the gradual shortening of flocculation time. Since the flocculation time of mixtures of split and native antitoxins varies according to the relative amounts of each (Table I), the flocculation time of an antitoxin undergoing

TABLE I

*Flocculation Time of Mixtures of Split and Native Antitoxic Pseudoglobulin*

Split pseudoglobulin (% of total)	0	10	20	50	80	90	100
Minutes	80	65	47	30	25	19	17

digestion will probably reach a minimum only when all the native molecules have been split.

Table II shows some of the results. When little pepsin was used, the flocculation time was at first considerably prolonged, and then decreased, as if the molecules acted upon by pepsin first passed

through a stage of denaturation before being either reduced to split antitoxin or completely destroyed. At about pH 4.5, this increase in flocculation time was the only change observed. It was still evident after three days, and was accompanied by no loss of antitoxic titer. Between pH 4.0 and 3.0, the loss in titer was about 20% in twenty-four hours; not much additional loss occurred in the next two days, even

TABLE II

*Hydrolysis of a 3.4% Solution of Native Antitoxic Pseudoglobulin in Water Acidified with HCl. Temperature 37°C.*

Exp. No.	Pepsin concen- tration		Time in hours					
			0	0.1	0.5	5	24	72
1	per cent 0.05	pH	4.0			4.1	4.2	4.3
		Antitoxin left (%)	100	100	100	95	95	90
		Flocculation (min.)	80	>180	>180	60	30	45
2	1.0	pH	4.1			4.3	4.4	4.5
		Antitoxin left (%)	100	95	95	85	80	75
		Flocculation (min.)	80	45	30	13	15	14
3	0.05	pH	3.5			3.8	3.9	4.0
		Antitoxin left (%)	100	100	100	95	85	80
		Flocculation (min.)	80	>180	135	15	10	14
4	0.05	pH	3.2			3.6	3.7	3.8
		Antitoxin left (%)	100	100	95	80	80	75
		Flocculation (min.)	80	ca 120	20	15	12	10
5	0.05	pH	2.6			3.1	3.3	3.4
		Antitoxin left (%)	100	35	30	30	30	30
		Flocculation (min.)	80	>180	50	20	12	13

when the pH remained below 4.0. Below pH 3.0, most of the antitoxin was rapidly destroyed.

Table III shows that the formation of nonprotein nitrogen followed closely the destruction of antitoxin with a slight lag due probably to the fact that the nonactive fraction of the molecule, which pepsin removes, is not completely broken down, but remains in part to form the s<sup>3</sup> component of Petermann (3). The percentage of tyrosine and

tryptophan decreased slightly with time, suggesting that these are among the first amino acids liberated in this type of hydrolysis.

When antitoxic pseudoglobulin that had been digested at pH 4.0 and purified by Pope's method (1, 11) was subjected to renewed

TABLE III

*Hydrolysis of a 3.4% Solution of Native Antitoxic Pseudoglobulin in Water Acidified with HCl. Pepsin Concentration 0.05%. Temperature 37°C.*

Time (hours)	0.5	1	2	4	7	24
pH	3.7				3.9	4.0
Nonprotein N (% of total N)	1.8	2.6	3.8	5.6	7.9	13.7
Tyrosine-tryptophan (% of N.P.N., calculated as tyrosine N)	3.4	3.7	3.6	3.5	3.2	3.0
Antitoxin destroyed (%)	5	5	10	15	15	20
Flocculation of antitoxin left (min.)	70	30	23	18	16	15

peptic action at about the same pH, under the same experimental conditions as those in Table II, there was hardly any hydrolysis. At pH 3.8, with a large amount of pepsin and concomitant dialysis to remove the products of digestion, only one third of the antitoxin was destroyed after two weeks at 4°C. (Table IV). In this experiment,

TABLE IV

*Hydrolysis of a 5.1% Solution of Split Antitoxic Pseudoglobulin in 0.2 M Acetate Buffer, 5% NaCl. Pepsin Concentration 6%. Temperature 4°C.*

Time (days)	2	5	14
	Per cent antitoxin destroyed		
pH 4.1 No dialysis	5	10	15
pH 3.8 Concomitant dialysis against 100 volumes same buffer	10	25	35

5% NaCl was added to prevent the formation of a precipitate between pepsin and antitoxin. These facts show that split antitoxin is appreciably resistant to peptic hydrolysis in moderately acid medium.

#### *Precipitation of Pepsin with Split Antitoxin*

Mixtures of crude pepsin and split antitoxin yield a precipitate when the pH of the solution is below about 5.0 and the concentrations of salt and of either reagent are sufficiently low. Precipitation is complete in a few minutes. When formed by slow dilution of a concentrated mixture, the precipitate is coarsely granular or composed of

spheroids and resembles a pepsin preparation that has just failed to crystallize.

The nature of the reaction was first studied with dilute solutions of either reagent, each acidified to pH 3.9 with HCl, and mixed in various proportions. Table V shows that when the amount of antitoxin was constant, maximum precipitation occurred with a crude pepsin:antitoxin ratio of 40 to 80 (tube 7 or 8). The point of equivalence, however, determined by adding more of either reagent to the supernatant after centrifugation, gave a ratio of about 4 (tube 3 or 4). When the amount of pepsin was constant, the pepsin:antitoxin ratio of maximum precipitation was much lower and was nearly the same as the equivalence point, again about 4 (tube 5 or 6). The complex became soluble in an excess of either reagent (tube 10). The point of maximum precipitation appeared to be also the point of most rapid flocculation, but this could not be clearly determined because the phenomenon was too gradual and spread over too many tubes. It depended definitely on total concentration and was further displaced toward the right when "constant antitoxin" titration was performed in a solution ten times more dilute.

If crude pepsin contains 20% pure pepsin (13), these data show that the complex formed contained nearly equal amounts of pure pepsin and antitoxin or, with molecular weights of 36,000 (13) and 116,000 respectively, about 3 molecules of pepsin to one of antitoxin. There was no evidence that this ratio depended grossly on the acidity since exactly the same results were obtained at pH 3.4. Below that there was danger of rapid hydrolysis, while above pH 4.5 the precipitate was too faint for convenient study. Attempts to repeat the same experiments with crystalline pepsin were unsuccessful because of the latter's partial insolubility in salt-free acid solution.

#### *Composition of Split Antitoxin-Pepsin Compound*

Large amounts of precipitate were obtained by mixing pepsin and antitoxin in approximately equivalent ratio in concentrated solution, acidifying with HCl to the desired pH, diluting with water and centrifuging the precipitate. The latter could be dissolved by the addition of a few drops of dilute sodium hydroxide and reprecipitated by acidification. It could also be washed and centrifuged repeatedly in dilute acid without significant loss. Little or no precipitate was obtained on dialysis of a concentrated mixture against dilute acid, since, as

TABLE V  
*Flocculation of Pepsin and Split Antitoxic Pseudoglobulin. Total Volume 1.0 ml. pH 3.9*

Tube No.	1	2	3	4	5	6	7	8	9	10
Antitoxin 0.1 mg.										
Pepsin (mg.)	0.06	0.13	0.25	0.50	1	2	4	8	16	32
Flocculation	±	+	+	++	++	+++	+++	++	+	±
Antitoxin added to supernatant	0	0	0	±	++	++++	++++	+++	+++	+++
Pepsin added to supernatant	++	++	+	0	0	0	0	0	0	0
Pepsin 1.0 mg.										
Antitoxin (mg.)	0.013	0.025	0.05	0.1	0.2	0.4	0.8	1.6	3.2	6.4
Flocculation	±	+	+	++	+++	+++	+++	+++	+++*	*
Pepsin added to supernatant	0	0	0	0	0	++	+++	+++	.	.
Antitoxin added to supernatant	+++	+++	+++	+++	++	++	±	0	0	

\* Precipitate did not sediment during centrifugation.

already mentioned, the compound was soluble in concentrated solution. As water was added to a concentrated mixture at constant pH, precipitation occurred over a wide range of dilution. This offered a means of isolating a series of fractions for study. Below a concentration of about 0.1% no additional precipitate formed but one-third at least of the compound remained in solution, as shown by the titration of the antitoxin in the supernatant after centrifugation. The addition of extra amounts of either reagent to the supernatant then failed to give a significant precipitate unless one of the reagents was originally in excess.

The precipitate formed at pH 4.4, 3.7 and 3.2 in slight excess of pepsin is analyzed in Table VI. Maximum precipitation took place at pH 3.7. As slightly less pepsin was bound by the antitoxin as the pH was decreased, the pepsin:antitoxin molecular ratio ranged from 3.0 to 2.3. At the same time, the antitoxin bound became poorer in active molecules, as shown by the increasing values of antitoxin N:Lf. Since this value in the original material was  $(175 \text{ mg.} \times \frac{14.7}{100}) : 3700 \text{ Lf}$  = 7.0  $\gamma$ , the antitoxin precipitated was in general of lower titer than the original solution. These differences are not considerable and the results show that, roughly speaking, pepsin is unsuitable for distinguishing clearly between active and inactive molecules.

TABLE VI  
*Precipitation of 100 mg. of Split Antitoxic Pseudoglobulin and 1 g. of Pepsin  
 in 220 ml. Water Acidified with HCl*

	pH 4.4	pH 3.7	pH 3.2
Protein precipitated (mg.)	80	124	55
Antitoxin recovered (%)	38	55	25
Pepsin:antitoxin molecular ratio	3.0	2.6	2.3
Antitoxin N per Lf ( $\gamma$ )	7.6	8.6	9.1

The increase in the amount of complex formed when pepsin was in excess, as indicated in Table V, was not studied in detail because of the inconveniently high dilution necessary to produce an appreciable precipitate under such conditions. When the complex thus formed was washed with dilute acid, dissolved with alkali, and reprecipitated with acid, some free pepsin remained in the supernatant. The precipitate, however, still contained an appreciable excess of pepsin, a fact attributable to the readiness with which pure pepsin, deprived of the other constituents of commercial pepsin, precipitates in acid solution.

The properties of fractions separated by progressive dilution are given in Table VII. The first fraction, which precipitated when the fluid volume was 0.3 l., was removed by centrifugation and washed in 0.001 M acetic acid. The supernatant was then diluted further and the next fraction removed. This step was repeated several times. Fraction 6 was obtained by adding two liters of dilute acid to another batch of the same materials, discarding the precipitate, and concentrating the supernatant by pervaporation. In such operations the total antitoxin recovery was about 80%, the deficit being probably due to some hydrolysis and to mechanical loss.

TABLE VII

*Gradual Precipitation of 1.75 g. of Split Antitoxic Pseudoglobulin  
and 10 g. of Pepsin at pH 3.9*

Fraction No.	0.001 M acetic acid added	Solu- bility limit	Protein precipi- tated	Pepsin: anti- toxin molecu- lar ratio	Anti- toxin re- covered	Antitoxin Titration			Antitoxin N	
						5/4 toxin excess	Opti- mal ratio	5/4 anti- toxin excess	In whole frac- tion	In floc- cules
	<i>Liters</i>	<i>pH</i>	<i>mg.</i>		<i>per cent</i>	<i>minutes</i>			<i>γ per Lf</i>	
1	0.3	5.6	395	2.1	9	65	30	60	10.6	1.39
2	0.5	5.2	415	2.2	11	34	26	34	8.7	1.35
3	0.9	4.8	367		11	26	21	27		1.24
4	1.7	4.5	582	2.6	18	26	18	26	6.7	0.94
5	4.0				4	18	15	18		1.14
6*	2.0				24	19	14	19		1.11

\* Fraction of complex still in solution after addition of 2.0 liters of dilute acid.

Column 3 of Table VII gives the pH of the dilute buffer in which each of the isolated fractions just failed to dissolve completely. It shows that the solubility of the fractions varied with the acidity as it varied with the dilution. Column 5 indicates that the more soluble fractions were those in which the pepsin:antitoxin ratio was highest. The table also shows that the antitoxin fractions thus separated differed appreciably in their serologic properties. The first fraction resembled undigested antitoxin in that its flocculation time was fairly long at optimal ratio, but was still much shorter than at a 4/5 or 5/4 optimal ratio. The other fractions flocculated over a broader range and with increasing rapidity. The amount of total antitoxin nitrogen

per Lf liberated by digestion is given in column 10. It is evident that the first fractions to precipitate were comparatively poorer in active antitoxin. In the last column the amounts of active antitoxin nitrogen associated with one Lf are given, as determined after flocculation with toxin by the method already used (6, 15). They show that the toxin-antitoxin complex contained less antitoxin nitrogen when the antitoxin was derived from the more soluble pepsin-antitoxin fractions. Calculated on the basis of all the antitoxin recovered, the average value was 0.00116 mg. N per Lf, the value already found for spontaneously formed split antitoxin (6).

#### DISCUSSION

The mode of action of pepsin and other proteolytic enzymes on antitoxic pseudoglobulin appears to be due to the peculiar ability of pseudoglobulin to yield, in the course of hydrolysis, a new protein of reduced molecular weight which still has all the characters of a native substance. This feature probably would have remained unknown longer but for the fact that the new protein carries antitoxic activity, so that its serologic properties were actually discovered before its physical characteristics were known. In addition to split antitoxin and nitrogenous material of low molecular weight, the peptic digest of antitoxin contains a heat-labile fraction which precipitates in salt-free solution at pH 6. Split antitoxin is only slowly hydrolyzed by pepsin in moderately acid medium and is thus able to form reversible compounds of appreciable stability with pepsin.

When dilute, salt-free and moderately acid solutions of pepsin and of split antitoxin are mixed in various proportions, they precipitate in a manner similar to antigen-antibody flocculation, especially to the protein-antiprotein rabbit-serum system, because of the existence of two distinct optimal flocculation zones (16, 17). If pepsin is compared to antibody, and antitoxin to antigen, the second optimal ratio is found in the zone of antibody excess, as is the case with antiprotein rabbit serum. Also, the pepsin:antitoxin molecular ratio of about 3 at the equivalence point is the same as the antibody:antigen ratio found in the combination of rabbit antibody with serum albumin or egg albumin (17). If the existence of two optimal ratios with antiprotein rabbit serum is related to the low solubility of the globulin fraction in which rabbit antibody is found, it should be noted that pepsin,

taking here the part of antibody, is slightly soluble in acid medium and remains dissolved in the presence of the other components of crude pepsin only as a supersaturated solution (13). The fact that pepsin-antitoxin precipitation can be demonstrated only in salt-free solution may of course suggest that the similarities just outlined are more apparent than real. They appear worth stressing, however, because of the parallel not infrequently drawn between antigen-antibody behavior and enzyme action (18, 19) and because the system studied here is probably the closest replica yet produced.

The split antitoxin-pepsin complex is markedly similar to the edestin-pepsin combination (7). In both cases, maximum precipitation occurs at about pH 4.0 and the two proteins combine with pepsin in approximately equal amounts. Both complexes are richer in pepsin when formed in the presence of excess pepsin. The antitoxin-pepsin combination is characterized by marked stability and reversibility, as shown by the unaltered toxin binding property of the antitoxin after it is released from union with pepsin. Furthermore, the compound is very soluble. As with edestin, the pepsin recovered after hydrolysis of the complex could be crystallized by Northrop's method (13). Fairly pure antitoxin was recovered after denaturation of the pepsin at pH 7.5 at 60°C. and its removal by 1/3 saturation with ammonium sulfate.

The information available permits no definite conclusion on the nature of the forces binding pepsin to other proteins. In the pH range of the present study, pepsin is an anion while antitoxin is probably present entirely as cations, so that an acid-base combination would be the simplest interpretation. But even with a pure protein such as crystalline edestin, the reaction could not be expressed on this assumption in simple mathematical terms (7). Such an interpretation would be even more difficult in the case of the pseudoglobulin studied, which cannot be crystallized without extensive fractionation (4) and whose heterogeneity is obvious. A recent investigation of the combination of pepsin with two plant viruses illustrates the complexity of the problem without shedding much light on the nature of the combination (20). The split antitoxin-pepsin compound has characteristics that should make it eminently suited to a closer physical study. Unfortunately, facilities for ultracentrifugation or electrophoresis were lacking, and the osmotic pressure method (9, 10) yielded only erratic

results, a phenomenon observed whenever the material under investigation is not perfectly stable.

The data in Table VII indicate not only that antitoxic activity was not equally distributed throughout the pseudoglobulin but also that the various fractions differed somewhat in their serologic properties. In the absence of more complete physical information, it may be temporarily assumed that the shorter flocculation time and the lower floccule nitrogen:Lf value of the antitoxin in the more soluble fractions were due to its consisting of slightly smaller molecules. These differences, however, could also be due to qualitative differences in the antitoxin such as would affect, for instance, the toxin:antitoxin molecular ratio in optimal proportions. It seems improbable that they were created, to any appreciable extent, during the precipitation of antitoxin with pepsin, since the pH was the same throughout and all fractions were handled in the same way. In general, the results reported show that pepsin does not lend itself appreciably, either in its binding of split antitoxin or in its hydrolysis of native antitoxin, to distinction between active and inactive molecules.

#### SUMMARY

The formation of split pseudoglobulin from native diphtheria antitoxic pseudoglobulin by peptic hydrolysis has been studied by following the decrease in antitoxic titer, the shortening of flocculation time and the appearance of nonprotein nitrogen.

Split antitoxin forms with pepsin, in moderately acid medium, a reversible complex of marked stability, most of which is insoluble in salt-free solution. This complex contains from 2 to 3 molecules of pepsin to one of antitoxin.

The split antitoxin-pepsin complex can be separated into fractions of increasing solubility. The antitoxin from each fraction differs in flocculation time with toxin and in nitrogen:Lf ratio.

The precipitation of pepsin and split antitoxin mixed in various proportions is somewhat similar to antigen-antibody flocculation.

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# The Effect of Concentrated Acid on Hair and Wool

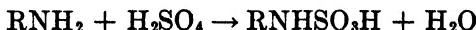
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Received January 11, 1945

## INTRODUCTION

The combination of keratin fibers with dilute acids is stoichiometric (0.80 millimol/g. wool) (1) and reversible on washing with water. On treatment with 80% sulfuric acid wool gained 5 to 8% in weight and the combined acid could not be removed on prolonged washing with water, dilute ammonia, sodium bicarbonate or triethanolamine (2). According to Harris *et al.* (3), concentrated sulfuric acid combines with the free amino and imino groups in the wool with the formation of sulfamic acid derivatives as shown by the following equation:



It seemed likely that hair and wool on treatment with concentrated sulfuric acid might undergo other or additional reactions and that other acids would react similarly. A study was made of the effect of concentrated sulfuric acid on the amino acid composition and solubility in thioglycolic acid of hair and wool.

Men's hair cuttings and virgin wool of 3-5 mm. lengths were washed with soap and after drying were extracted with ether and chloroform for eight hours each. The procedure adopted with concentrated sulfuric acid was as follows: thirty parts by weight of the acid, cooled to - 5°C., were added to the loosely packed, dry, cold fiber. After standing at zero degrees for twenty-four hours, the mixture was cooled to - 5°C. and the bulk of the uncombined acid poured off. The treated fiber was then slowly added to 3000 g. of crushed ice with thorough and vigorous mixing. During this procedure the temperature remained under 5°. The hair-ice water mixture was filtered through cheese cloth and the residue washed thoroughly with ice water. The total nitrogen of the pooled acid and washings was determined. After removing the free sulfuric acid in running water, the treated fiber was air-dried and extracted with ether for eight hours. The bound acid was determined on an aliquot as barium sulfate after hydrolysis with 6 N hydrochloric acid.

Experiments with different hair samples have shown that under the above conditions 1-2% of the total hair nitrogen is dissolved in the

sulfuric acid and washings. Treatment up to five days at zero degrees increases to 3-4% the amount of nitrogen going into solution without altering the amount of sulfuric acid bound to the fiber. In red hair, the amount of bound sulfuric acid found (9.6-10.6%) was lower than that in dark hair (12-14.5%), indicating differences in chemical composition.

#### SOLUBILITY OF NORMAL AND ACID TREATED KERATINS IN THIOLYCOLIC ACID

The solubility of hair containing 12.4% bound sulfuric acid in sodium thioglycolate solutions of different pH was studied, using untreated hair for comparison. Goddard and Michaelis (4) found that wool does not dissolve in thioglycolic acid at pH 9 and only slowly at pH 10, while 60% goes into solution at pH 11-12. Alkali alone of the same pH had no dissolving action. Also, removal of the thioglycolate from the keratin solution by dialysis, or acidification, caused the precipitation of the protein, which, when dry, was difficultly soluble in free alkali.

Normal hair (2.5 g.) and sulfuric acid-treated hair (2.5 g.) were mixed with 50 ml. of *M/2* thioglycolate solution of varying pH, allowed to stand one hour and then shaken for three hours. The mixtures were pressed out, filtered and the extraction repeated in the case of normal hair. The pH of each solution was determined with a glass electrode before and after treatment of the keratin. The pH of the solutions after extraction was used in tabulating the data. The extracts were centrifuged and their nitrogen content determined. An example of the amount of nitrogen going into solution in the first and second extract of normal hair and the first extract of sulfuric acid-treated hair is given in Table I.

Normal hair shows little solubility up to pH 8.25, the minimum occurring at or near the iso-electric point. The introduction of acid groups into the keratin increased the solubility markedly, especially at lower pH, *e.g.*, at pH 8.27 in two extractions only 2.01% of the untreated hair nitrogen went into solution, whereas at pH 8.37 57% of the sulfuric acid-treated hair nitrogen was soluble. Experiments with normal and sulfuric acid-treated wool gave similar results.

To determine the effect of the treatment with acid on the solubility of the kerato-protein, the thioglycolate was removed by dialysis against water and buffer solutions of different pH. In the case of thioglycolate extracts of normal hair and wool, dialysis against water, phosphate and borate buffers up to pH 8.7 caused the precipitation

TABLE I

*Solubility of Normal and H<sub>2</sub>SO<sub>4</sub>-Treated Hair in Thioglycolate Solutions of Varying pH*

Normal Hair			H <sub>2</sub> SO <sub>4</sub> -Hair		
1st Ext.	pH	Protein N per cent	2nd Ext.	pH	Protein N per cent
	2.14	1.06		2.07	1.42
	4.33	0.82		4.33	0.67
					5.02
	8.27	1.10		8.25	0.91
	9.22	6.63		9.22	6.70
*	10.00*	13.0		10.00	10.8
	10.42	16.95		10.35	13.08
	12.35	37.7		12.50	37.06

\* Extrapolated from curve.

of protein, while after dialysis against borate buffer of pH 9.3 most of the protein remained in a strongly opalescent solution. After dialysis of the extracts from sulfuric acid-treated hair and wool, the keratin remained completely soluble. The proteins precipitated on addition of acid to pH 3.5–5.0. The moist precipitate was easily redissolved in water on addition of ammonia to pH 6 or higher. Repeated solution and precipitation did not alter the bound sulfate content of the soluble keratin.

Experiments were also carried out on the effect of acetic, formic, lactic and phosphoric acids on hair and wool. The temperature and duration of treatment, which varied from 0°–40° and from 1–8 days, were such that only 1–5% of the protein nitrogen dissolved in the acid used. Hair and wool bound less phosphoric acid and organic acids than sulfuric acid, e.g., wool treated for 18 hours at 38° with 85% phosphoric acid contained 1.5% PO<sub>4</sub>, hair treated with 90% formic acid at 40° for six days contained approximately 1.0% formic acid. Keratin solutions were prepared by extraction of the treated fiber with sodium thioglycolate of pH 10 and dialysis, as previously described. The proteins were precipitated by addition of acetic acid between pH 6.5–4.0.

#### EFFECT OF ACID TREATMENT ON AMINO ACID COMPOSITION

To study the effect of the experimental procedure on the keratin, the amino acid composition of hair was determined before and after

treatment with sulfuric acid. To assure uniform results hair (red) of only one person was collected. During its growth the hair underwent no bleaching, dyeing or permanent waving. On treatment with concentrated sulfuric acid as described above, 1.9% of the hair-N went into solution. The remaining hair contained 10.23% bound sulfuric acid.

*Methods:* For each analysis the samples were brought to constant weight by drying in vacuum over  $P_2O_5$  at 60°. The ash content, the nitrogen by micro-kjeldahl analysis, and the total sulfur, gravimetrically as  $BaSO_4$ , following the wet oxidation procedure of Jones (5), were determined in treated and untreated hair. The bound sulfuric acid was determined directly as barium sulfate after 20 hours hydrolysis with 6 N HCl and was calculated as per cent sulfate or sulfate-S. The total sulfur minus the cystine- and bound sulfuric acid-S gave the organic non-cystine sulfur.

For the determination of the amino acids, hair was hydrolyzed with 30 times its weight of 6 N HCl for 24 hours. The hydrolysate was brought to pH 5.5-6.0 by careful addition of  $Na_2CO_3$  with stirring. After standing overnight the humin was centrifuged off, washed and its nitrogen content determined. The filtrate and washings were decolorized by activated charcoal (Merck), made up to volume and aliquots taken for the analyses. For colorimetric determinations the Cenco-Sheard spectrophotometer was used at the wave lengths given. Cystine was determined by the

TABLE II

*S and Amino Acid Distribution in Untreated and Sulfuric Acid-treated Red Hair*

	Normal per cent	$H_2SO_4$ -treated per cent
Ash	0.415	0.856
N-content	15.50	14.05
Total-S	5.24	8.41
$SO_4$ -S	—	3.51
Organic-S	5.24	4.90
Cystine-S	4.46	4.08
Organic Non-Cystine-S	0.78	0.82
N: organic-S	2.96	2.87

*Amino Acid-N Distribution in Per Cent of Total Nitrogen*

Humin-N	0.64	1.26
Cystine-N	12.66	12.56
Arginine-N	19.31	14.11
Histidine-N	1.59	1.67
Threonine-N	5.29	5.31
Serine-N	6.30	5.70
Tryptophane-N (Folin-Lugg)	0.33	0.26
Tryptophane-N (Eckert)	0.30	0.24
Tyrosine-N (Acid Hydrol.)	1.31	0.88
Tyrosine-N (Alkal. Hydrol.)	1.45	0.95
Phenylalanine-N	1.99	1.73

Folin-Lugg method (650 mu) applied both to the hydrolysate and to the copper precipitate of the mercaptan (6, 7). The latter gave the more consistent results, which were used in the table. Threonine and serine were determined by the methods of Nicolet and Shinn (8). MacPherson's (9) modifications of the diazotization reaction for histidine (495 mu) and the Sakaguchi reaction for arginine (495 mu) were used.

For the determination of tyrosine, tryptophane and phenylalanine, hair samples were hydrolyzed with 5 N NaOH for 24 hours. Tyrosine and tryptophane were determined by the method of Folin-Lugg (10). Tyrosine was also determined in the acid hydrolysate (500 mu) after precipitation of tryptophane. When the latter procedure was omitted, low values were obtained in some analyses. The tyrosine results obtained on the acid and alkaline hydrolysates were in good agreement. Tryptophane by Lugg's method (425 mu) gave widely varied results. After as little time as 45 seconds the color begins to fade. The method of Eckert (11) (550 mu) gave reproducible results. Phenylalanine was determined by the method of Kappeler-Adler as carried out by Block and Bolling (12) (540 mu). The blank reading was obtained on the same aliquot used for the analysis after discharging the color with solid sodium hydrosulfite. This modification gave reproducible values on protein hydrolyzed with and without addition of phenylalanine. For each amino acid three analyses were carried out on duplicate hydrolysates, with the necessary controls. All nitrogen and sulfur values were calculated for ash-free protein. For comparing the amino acid composition before and after treatment with sulfuric acid all values are given as amino acid-nitrogen in per cent of total nitrogen (Table II).

## RESULTS AND DISCUSSION

The treatment with sulfuric acid did not affect the cystine, histidine and threonine content of the hair. Serine showed a 10% decrease and nearly 25% of the arginine was destroyed by the treatment. Blackburn and Phillips (13) found a similar loss of arginine in acetylated wool. The aromatic amino acids tryptophane, phenylalanine and especially tyrosine have shown a decrease. This decrease may be accounted for in part by sulfonation of the benzene ring. These sulfonated compounds are not precipitated by barium chloride and, therefore, are not included in the determination of bound sulfuric acid. This could account for the approximately 5% increase in non-cystine organic sulfur found in treated hair. Ash and humin nitrogen were increased.

These results indicate that when keratin fibers are treated with concentrated sulfuric acid at low temperatures, chemical reactions take place in addition to that of sulfamic acid formation. With other acids similar reactions occur. Such treatment brings about changes in the chemical and physical properties of the kerato-protein which are of technological interest since treatment with acids is applied in the

carrotting of felt and the carbonization of wool. The possibility of preparing neutral soluble keratins of high molecular weight may be of importance in the preparation of artificial fibers.

### SUMMARY

- (1) Acid groups can be introduced into keratin fibers by treatment with concentrated acids.
- (2) The treated fiber yields on extraction with alkaline thioglycolate, followed by dialysis, a protein which is soluble at pH 5-6 or higher.
- (3) Treatment with sulfuric acid apparently does not affect the cystine, histidine, or threonine content, causes a slight decrease in serine and phenylalanine, and a marked loss of arginine, tyrosine and tryptophane of the keratins.

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# Fractionation of Hair, Chemical and Physical Properties of the Hair Fractions

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Received January 11, 1945

## INTRODUCTION

Histologically hair is composed of three different cell structures:

1. The cuticle, a thin outer layer of overlapping scales.
2. Cortex, spindle-shaped, elongated cells, forming the main body of the hair.
3. Medulla, a continuous band of hollow cells in the axis of the hair shaft, which is often partly or completely missing. In addition, pigment granules, cell nuclei and submicroscopic fibrils (1) have been observed in the cortex. The presence of an intermediate membrane between the cuticle and cortex has been assumed (2). The relation of the histological structure to the chemical properties and reactions of hair and wool is generally recognized. Because of the difficulties involved in the separation of hair or wool into its structural components, chemical investigations are usually restricted to the whole fiber (3).

According to some investigators (4) neither histidine nor tyrosine is present in the cuticle as color reactions for these amino acids are negative when carried out on undamaged wool. King and Nichols (5) concluded from their investigations that the sulphur content of the keratin fiber decreases from the outside to the inside of the hair shaft, the cuticle having the highest amount. In agreement with this, Lloyd and Marriot (6) found that alkali-insoluble medulla of goats hair contains 0.23% sulfur compared to 3.60% in the total fiber. Chamberlain (7), however, reported no difference in the sulfur values of natural hair and the same hair descaled by mechanical scraping. Wool, chemically modified by reduction with thioglyeolic acid followed by alkylation with ethyl bromide, yielded, after pepsin digestion, 2.3% insoluble scales (cuticle) (8). The scales had 60% more sulfur, 18% more serine and only 50% of the tyrosine and arginine content of the untreated wool.

A method has been developed for the separation of cuticle and the fractionation of the descaled hair. The chemical and physical properties of the isolated fractions and their amino acid composition have been studied. The changes produced by the method employed were also investigated.

### I. FRACTIONATION OF HAIR KERATIN

Men's hair cuttings were washed with soap and water, dried, and then extracted for eight hours each with ether and chloroform. The dried hair in portions of 100 g. was treated with concentrated sulfuric acid at 0°C. for 24 hours. The method and the chemical changes brought about by this treatment have been reported in a previous paper (9).

Less than 2% of the hair nitrogen was soluble in the sulfuric acid. After dialysis, most of the nitrogen of the sulfuric acid solution was lost, indicating that only a part of the soluble material was in protein form.

**Cuticle.** Cuticle can be almost quantitatively removed from the sulfuric acid-treated hair by repeated shakings with water. The hair in 600 ml. of water was shaken mechanically for three hours, filtered through gauze, and the cuticle freed from contaminating hair particles by repeated decantations. The shaking causes the hair residue to form small twisted balls which were pulled apart, resulting in liberation of more cuticle. The entire process was repeated until the hair was practically cuticle-free. This required from 14 to 18 shakings for each experiment. Each cuticle fraction was washed with water until acid free (the washings saved), then dried with acetone and ether.

Each cuticle preparation was examined under the microscope and the percentage of cortex cells estimated. Preparations of equal purity were united. The first third of the cuticle preparations usually contained less than 1%, the second third had 1 to 5% and the last third had 6 to 30% cortex cells. The first 8-10 fractions contained from 400 to 1100 mg. of cuticle, whereas the last fractions contained only 150 to 200 mg. For the analyses, cuticle containing less than 1% of cortex cells was used.

The first cuticle fractions also contained pigment granules which were easily separated from the cuticle by slowly centrifuging for short periods of time. Only in the case of red hair-pigment were the granules soluble in dilute ammonia.

**Cuticle Washings.** The first washings of cuticle fractions 1 and 2 were strongly opalescent and contained significant amounts of protein. Only these washings were dialyzed as all others were low in nitrogen. After dialysis the solution was filtered, concentrated *in vacuo* to a small volume and the protein precipitated with acetic acid at pH 3.5-4.0.

The protein present in the sulfuric acid and in the cuticle washings after dialysis gave a weak to negative biuret reaction, which became positive on prolonged standing, on preheating for 5 minutes at 100°

in presence of alkali, and after addition of guanidine carbonate. Since the biuret reaction indicates the presence of two amino groups, one of which must be unsubstituted, and linked by an N- or C-atom, these results suggest that the amide groups in these keratin fractions are inaccessible to the biuret reagents. Addition of guanidine or treatment with alkali changes the rigid steric configuration of the keratins in a way similar to that observed in other proteins in regard to sulphydryl and phenol groups (10).

*Cuticle-Free Hair.* The remaining hair, stained with methylene blue, showed microscopically few cuticle cells sticking to the hair shaft. A sample of this fraction was removed for analysis.

*Cortex Acid-Insoluble.* The cuticle-free hair was extracted with  $3 \times 1$  liter portions of 0.5 M sodium thioglycolate of pH 9.8. The last extract contained only a small amount of nitrogen. The combined extracts were dialyzed until disappearance of the cobalt sulfate reaction for thioglycolate, filtered, and brought to pH 3.5-4.0 with acetic acid. After standing overnight, the precipitate was centrifuged off, washed with 0.5% acetic acid and dried with acetone and ether. The dried protein powder is water-insoluble. It can be brought back into solution with 1% ammonia, and a neutral solution can be obtained either by dialysis or addition of acid. The protein can be precipitated again by acidification.

*Cortex Acid-Soluble.* The filtrate and washings from the preparation of the cortex acid-insoluble fraction were concentrated *in vacuo* below 35°C., dialyzed, precipitated with acetone and dried with acetone and ether. This fraction dissolves readily in water.

*Residue.* The insoluble hair residue, after the above extraction, was washed in running water until the bulk of the sodium thioglycolate was removed. To remove the residual thioglycolate and any soluble protein in the swollen hair residue, use was made of an observation by Goddard and Michaelis (11) that thioglycolate-soluble keratin is digested by pepsin. The residue was digested at 40°C. with a 0.1% pepsin solution in HCl of pH 1.8 in the presence of chloroform. The progress of the digestion was followed by determining the nitrogen content of the solution which reached a maximum in four days. After six days, the pepsin digestion of the residue was repeated, whereupon only a small amount of additional non-protein nitrogen went into solution. The undigested residue was washed in running water until it gave a negative reaction for thioglycolic acid, and then dried with

acetone and ether. It consisted of grayish weak fibers, which, under the microscope, were composed of medulla surrounded by a part of the cortex, and whose diameters were equivalent to 0.25 to 0.5 that of the original hairs.

This fractionation was carried out on portions of the same hair and on hair of different color. The amounts found are approximate, since from 10 to 20% of the original hair nitrogen was lost during the procedure. Table I summarizes the values found in eight experiments.

TABLE I  
*Hair Fractions in Per Cent of the Total Hair*

$\text{H}_2\text{SO}_4$ -soluble	1.2- 2.2
Cuticle, washings	1.5- 2.8
Cuticle	6.9-10.5
Cortex, acid-soluble	3.0- 5.0
Cortex, acid-insoluble	52.0-60.0
Pepsin digestible	7.4- 8.5
Residue	10.8-14.0

The results indicate that cuticle forms a larger part of the hair than has generally been assumed. Most of the cortex is soluble in alkaline thioglycolate. The inner part of the cortex and the medulla apparently are insoluble in thioglycolate and are not digestible by pepsin. Other investigators have observed microscopically a difference in behavior between the inner and outer part of the cortex toward sulfuric acid (12) and Pauly's diazo reagent (13).

## II. X-RAY AND ELECTROPHORETIC STUDIES

X-ray investigations by Wood (14) on samples containing 80% wool cuticle have indicated that the degree of crystallinity and alignment of crystallites in the scale cells is poor compared with that in the cortex. In our experiments an X-ray study has been made on a preparation containing more than 99% cuticle. The films, for which we are indebted to Dr. I. Fankuchen of the Brooklyn Polytechnic Institute, showed one halo at about 0.5 Å.U. and blackening about the central part, indicating a non-crystalline structure of the cuticle.

The availability of a keratin in soluble form representing the greater part of the hair made possible the determination of its mobility and uniformity in the electric field. An electrophoretic investigation at various pH values was carried out on the cortex acid-insoluble and

cortex acid-soluble fractions of sulfuric acid treated hair containing 7.2 and 6.6% bound sulfuric acid respectively.

These data were compared with those obtained on fractions prepared from untreated hair, and from hair treated with 90% formic acid for 7 days at 40°C. and washed free of the acid. Both of these were extracted with thioglycolic acid at pH 9.8. The thioglycolate extract of untreated hair was dialyzed against a borate buffer of pH 9.3. The resulting solution was strongly opalescent. (*Normal Hair-extract*).

The protein of the thioglycolate extract of the formic acid-treated hair remained in solution on dialysis against running water. It was precipitated by adjusting to pH 5 with acetic acid, redissolved in weak

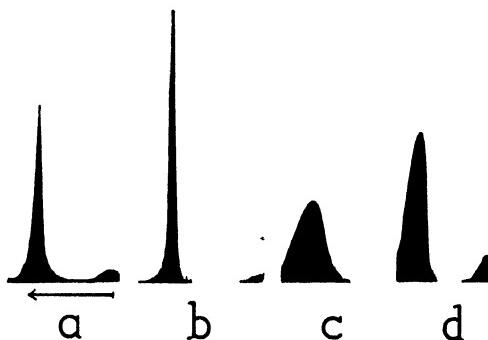


FIG. 1

Electrophoretic Patterns (Descending) of (a) Normal Hair Extract, (b) Formic Acid-Acid Insoluble Fraction, (c) Cortex, Acid-Soluble Fraction, and (d) Cortex, Acid-Insoluble Fraction.

ammonia and the solution dialyzed until ammonia-free. (*Formic acid-acid insoluble*). The protein of this solution after analysis contained 1% formic acid.

*Results.* All of these preparations yielded only a single electrophoretic component (See Fig. 1 and Table II). The introduction of 7.2% H<sub>2</sub>SO<sub>4</sub> increased the mobility from 7.1 to  $9.4 \times 10^{-6}$  cm.<sup>-2</sup> volt<sup>-1</sup> sec.<sup>-1</sup> when examined at pH 9.3 under the same conditions. The introduction of 1.0% formic acid did not, however, change the mobility appreciably.

It was of interest to find that the mobility of the cortex acid-soluble fraction (the only one which could be studied over a wide pH range) was not appreciably affected by pH and that the protein was still

TABLE II  
*Electrophoretic Mobilities of Hair Protein Preparations*

Preparation	pH	$u \times 10^4$
Normal hair extract	9.3 B*	-7.1a†
Formic acid-acid insoluble	9.3 B	-6.9b
	7.4 P	-6.4
Cortex, acid-soluble (6.6% SO <sub>4</sub> )	1.0 G	-7.0
	2.0 G	-6.5
	3.0 G	-9.9
	4.0 A	-9.4
	7.4 P	-9.3c
Cortex, acid-insoluble (7.2% SO <sub>4</sub> )	6.0 P	-9.3
	7.4 P	-9.2d
	9.3 B	-9.4

\* B = 0.18 M Borate + 0.01 M NaOH + 0.1 M NaCl

P = 0.02 M Phosphate + 0.15 M NaCl

G = 0.1 M Glycine + HCl + NaCl

A = 0.05 M Acetate + 0.05 M NaCl

† See fig. indicated.

negatively charged at pH 1.0. This fraction was completely soluble down to pH 3.0, but at pH 2.0 and below it was only partially soluble. It is assumed, therefore, that this protein does not have a definite isoelectric point. Nevertheless, it may have an isoelectric zone in the region below pH 1.0.

### III. CHEMICAL COMPOSITION OF THE KERATIN FRACTIONS

The fractions analyzed were (1) cuticle, (2) cuticle-washings, (3) cuticle-free hair, (4) cortex acid-soluble, (5) cortex acid-insoluble, and (6) residue. They were prepared as already described from a single mixture of men's brown hair.

*Methods.* In all fractions the ash content, nitrogen, total sulfur and the bound sulfuric acid were determined. The results were calculated for ash-free material.

Humin-N, serine, threonine, arginine, histidine, tyrosine, tryptophan and phenylalanine were determined in all fractions except the cuticle-washings. Cystine was determined in all fractions. All values were calculated as amino acid nitrogen in per cent of total nitrogen. Cystine-S was calculated from the cystine values. Total S minus cystine-S and bound sulfuric acid-S gave organic non-cystine-S. The methods used, with their modifications, are described in a previous paper (9). The data are given in Table III.

**Results.** Cuticle and cuticle-free hair showed a high ash content. The cortex acid-insoluble fraction had a low ash, while the ash content of the cortex acid-soluble fraction was especially high and remained high after redissolving in acetic acid and dialysis.

The organic, non-cystine-S of cuticle and cuticle-free hair are in

TABLE III

	Cuticle	Cuticle—washings	Cuticle free-hair	Cortex acid-soluble	Cortex acid-insoluble	Residue
<i>Ash, N, and S Content in Per Cent</i>						
Ash	2.330	2.349	2.368	5.045	0.132	0.128
N-content	14.57	14.83	14.33	14.38	15.26	14.18
Total Sulfur	10.70	7.53	8.53	8.58	7.71	6.84
SO <sub>4</sub> -Sulfur	4.78	2.43	3.47	2.18	2.40	2.28
Cystine-Sulfur	5.05	3.85	4.28	3.90	4.17	3.11
Non-cystine Organic Sulfur	0.87	1.25	0.78	2.46	1.14	1.45
<i>Amino Acid Nitrogen in Per Cent of Total Nitrogen</i>						
Humin-N	1.62	—	0.61	0.38	0.54	0.28
Cystine-N	14.15	11.32	13.03	11.88	11.92	9.56
Arginine-N	8.79	—	16.78	10.03	17.40	17.21
Histidine-N	1.08	—	1.80	1.17	2.25	2.35
Threonine-N	3.09	—	5.65	5.10	5.25	4.61
Serine-N	6.78	—	5.30	2.03	3.08	4.25
Tryptophan-N (Folin-Lugg)	0.16	—	0.29	0.30	0.23	0.30
Tryptophan-N (Eckert)	0.18	—	0.24	0.26	0.24	0.25
Tyrosine-N	0.72	—	0.95	0.74	1.02	1.13
Phenylalanine-N	0.88	—	1.35	0.95	1.19	1.89

close agreement with and similar to values reported for red hair (9) and normal wool (15). Methionine would account for about 20–25% of this sulfur.

In the cuticle, cystine is 8.5% and serine is 30% higher than in the cuticle-free hair, while threonine is 55%, tyrosine 77%, tryptophan 76%, histidine 72%, and phenylalanine 66% lower.

The chemical composition of the cortex acid-insoluble, cortex acid-soluble and residue was influenced by treatment with thioglycolate.

The sulfate-S values of the cortex fractions and residue indicate that thioglycolate of pH 9.8 has removed 20% of the bound sulfuric acid from the cuticle-free hair. The low cystine content of these fractions might be due to the method of preparation, since the cysteine formed by the action of thioglycolate is attacked by alkali. No cysteine was found in any fraction. After treatment with thioglycolate, threonine and serine contents were lower, and from the work of Nicolet *et al.* (16) it is probable that this decrease is due to action of the alkali.

The composition of cortex acid-insoluble, cortex acid-soluble, and residue may be compared to that of cuticle-free hair. The organic sulfur and cystine were lowest in the residue, which also showed the highest value for serine, tyrosine and especially phenylalanine. Arginine, histidine, tyrosine and phenylalanine were lowest in the cortex acid-soluble.

#### DISCUSSION

Histological and X-ray studies have shown that hair has a heterogeneous structure. The present study presents evidence that hair is made up of keratins which differ in chemical composition and physical properties. The method employed involved chemical changes, the investigation of which made it possible to differentiate between properties acquired during the treatment and those inherent in the fractions. Physical methods of separation would have offered no advantage, inasmuch as powdering of keratins (17) brings about changes in physical and chemical properties and causes destruction of cystine.

The isolation of pure cuticle made it possible to establish its non-crystalline structure and its difference in chemical composition from the remaining fiber.

The protein found in the cuticle washings may be related to the intermediate membrane present between cuticle and cortex.

From the cortex, two fractions were obtained by extraction with alkaline thioglycolate which differed in their physical properties and chemical composition but which behaved similarly in the electrophoretic field. Both fractions were digestible by pepsin. The inner part of the cortex, which also contains the medulla (residue), was insoluble

in thioglycolate and was not digestible by pepsin at pH 2. This fraction also differed in chemical composition from the soluble keratins. The cystine content of the residue and cortex acid-insoluble is not likely to be the cause of the differences in their behavior since it is almost the same in these fractions.

Not only were variations in chemical composition among the hair fractions observed, but also differences in the behavior of keratins compared to other proteins. Cuticle, which contains histidine and tyrosine, gave negative color reactions for these amino acids in the intact fiber and after isolation, while descaled hair and wool gave positive color reactions for these amino acids. In aqueous solution, some keratin fractions gave a weak or negative biuret reaction, which became positive on denaturation. These findings suggest that the difference in behavior between keratins and other proteins is not based only on the fibrous state and insolubility of keratins.

Treatment with sulfuric acid resulted in formation of a sulfamic acid and partial sulfonation of aromatic amino acids. (9). The bound acid is not removed from the keratin by prolonged washing with water and dilute alkali. The finding that alkaline thioglycolate removed 20% of the bound sulfuric acid from cuticle-free hair would indicate a different reactivity of the sulfamic acid group dependent on its linkage to the protein or the presence of another linkage between the keratin and sulfuric acid.

#### SUMMARY

A method has been worked out for the isolation from sulfuric acid-treated hair of kerato-proteins which differ in their chemical composition and properties.

A single cuticle fraction was obtained, which contained tyrosine and histidine, although color reactions for these amino acids were negative when carried out on normal hair and isolated cuticle. The non-crystalline structure of cuticle was established.

From the cortex two fractions were obtained by extraction with alkaline thioglycolate which differed in chemical composition. Each fraction yielded a single electrophoretic component which had the same mobility.

The inner part of the cortex and the medulla were insoluble in alkaline thioglycolate and were not digestible by pepsin.

The bound sulfuric acid could be partially removed by alkaline

thioglycolate but not by alkali alone. The relation of the experimental results to keratin structure is discussed.

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# Investigations of Amino Acids, Peptides and Proteins.

## XXII. Percentages of Some Amino Acids in Lactobacilli\*

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Received April 24, 1945

### INTRODUCTION

Bacteria vary markedly in chemical composition according to the experiments of early investigators,<sup>1</sup> and Nencki (3) concluded in 1884 that these differences are greater for related forms of bacteria than for other orders of plants and animals. Conclusions from these early experiments are not highly reliable because, as has been pointed out by Leach (2), the microorganisms were grown under widely varying conditions on media containing natural food materials of variable and uncertain composition. Although this problem is of considerable importance because of the role of microorganisms in animal nutrition and the possible relation of the chemical composition of bacteria to the evolution of living forms, the writers' interest is limited, at present, to studies of amino acids in bacterial cells. In the past, this has been difficult because of the lack of convenient methods for the determination of amino acids with satisfactory accuracy in the relatively small quantities of bacterial cells available for study. At the present time, relatively simple and reasonably accurate microbiological procedures for the assay of amino acids are available for this purpose.

Amino acids in bacteria were first determined by Leach (2). *Escherichia coli* cells were hydrolyzed with sulfuric acid and the basic amino acids were determined by

\* This work was aided by grants from the Gelatin Products Corporation, Merck and Company, Inc., The Nutrition Foundation, Schering and Glatz, and the University of California. The authors are indebted to W. Frankl, B. Merrifield and S. Shankman for technical assistance. The material in this paper was taken in part from a thesis submitted by M. N. Camien in partial fulfillment of the requirements for the M.A. degree in Microbiology, University of California, Los Angeles. For Paper XXI in this series see Dunn, *et al* (1).

<sup>1</sup> The literature prior to 1905 has been reviewed by Leach (2).

the Kossel and Kutscher procedure. Lysine was isolated as its picrate and hydrochloride but neither histidine nor arginine was definitely identified. Amino acids in bacteria were first investigated extensively by Tamura (4-6) who isolated leucine and tyrosine as the free amino acids, arginine and histidine as their picrolonates, and leucine, isoleucine, proline and valine as their copper salts from the sulfuric acid hydrolysates of *Mycobacterium tuberculosis*,<sup>2</sup> *Corynebacterium diphtheriae*, and *Mycobacterium lacticola*. The last microorganism was grown on a synthetic medium containing ammonium lactate and asparagine as the sole source of nitrogen. The presence of tryptophane was indicated by a color reaction and the identity of the other amino acids was established by nitrogen analysis. About the same time, Omeliansky and Sieber (8) determined arginine, histidine and lysine in *Azotobacter chroococcum* by the Van Slyke nitrogen distribution procedure. Later investigators determined these and other amino acids in *Mycobacterium tuberculosis* (9-14), *Bacterium lactis aerogenes* (15), *Escherichia coli* (16), *Azotobacter vinelandii* (17, 18), *Azotobacter agilis* (17), *Azotobacter beijerinckii* (17), and *Azotobacter chroococcum* (17) by analogous procedures.

The bacteria employed in the present investigation were *Lactobacillus arabinosus* 17-5,<sup>3</sup> *Lactobacillus casei*,<sup>4</sup> *Lactobacillus fermenti*,<sup>5</sup> *Lactobacillus pentosus* 124-2,<sup>6</sup> and *Escherichia coli* (communior).<sup>7</sup> The last microorganism was included because it is capable of growth on an amino acid-free medium. Each bacterium, except *Lactobacillus fermenti*, was grown on two synthetic media of different composition.

## EXPERIMENTAL

The media were prepared fresh as required. The same stock solutions of vitamins, salts, purines and hydrolyzed casein employed previously in investigations with *Leuconostoc mesenteroides* P-60 (19, 20) were utilized. The final solutions, adjusted to pH 6.8, were made to volume and autoclaved 10 to 15 minutes at 15 pounds pressure in round-bottom flasks of 2 to 12 l. capacity. A small quantity of flocculent precipitate (probably iron phosphate) which formed during autoclaving was removed by aseptic filtration with a large Mandler candle.

The media (20 to 100 ml. aliquots) were inoculated with stock cultures of the microorganisms carried on Bacto-yeast dextrose agar and the suspensions, after incubation for 24 hours at 35°, were added to the nutrient media (1 or 8 l. volumes). The lactobacilli were incubated 24 to 30 hours at 35° and *Escherichia coli*, which is

<sup>2</sup> Investigations of amino acids in tubercle bacilli have been reviewed by Wells and Long (7).

<sup>3</sup> American Type Culture Collection, No. 8014.

<sup>4</sup> American Type Culture Collection, No. 7469.

<sup>5</sup> American Type Culture Collection, No. 9338.

<sup>6</sup> Obtained from the University of Texas through the courtesy of Dr. E. E. Snell.

<sup>7</sup> Obtained from the Department of Bacteriology, University of California, Los Angeles.

slow growing under the stipulated conditions, was incubated for 48 to 60 hours at 35°. Aseptic technique was employed throughout.

At the end of the incubation period the cultures were thoroughly chilled in the refrigerator and the temperature was maintained as low as possible during the subsequent operations in order that autolysis might be minimized. The cultures were centrifuged, suspended in normal saline and re-centrifuged. This process was repeated twice with normal saline, once with 50% ethanol and twice with 95% ethanol and diethyl ether. Each suspension was shaken for 10 minutes before centrifuging. The final products, dried over night at 60 to 70°, were very fine, nearly white powders. Microscopic examination revealed intact cells with no evidence of cell disintegration.

Acid hydrolysates for the determination of amino acids other than tryptophane were prepared by refluxing the cell preparations for 20 hours in 10 volumes of 8*N* hydrochloric acid. Alkaline hydrolysates for the determination of tryptophane were prepared as described by Greene and Black (21).

Total nitrogen in the cell preparations was determined by a semimicro-Kjeldahl procedure.<sup>8</sup> The amino acids in the hydrolysates were determined by the authors' published (1, 20, 22, 23) and unpublished microbiological assay procedures. Since some of the bacterial cells contained both *d*(-)- and *l*(+)-glutamic acid, the glutamic acid assay procedure was modified to permit determination of both optical forms of this amino acid. The proportions of the isomers were estimated by comparing the acid produced at various levels of the hydrolysate with that observed in media containing from 10:1 to 5:3 proportions of the *l*(+)- and *d*(-)-antipodes. These standard curves fell between those shown on page 601 of a previous paper (23). Although the proportions of isomers can be measured only approximately, it is probable that the total glutamic acid can be determined more accurately by this procedure in solutions containing both isomers than by the original method. Tryptophane was determined in the alkaline hydrolysate by the microbiological assay procedure of Greene and Black (21, 24). It was found that 77% of the natural tryptophane present initially in a mixture of this and other amino acids remained after the mixture had been autoclaved with barium hydroxide under the conditions employed by Greene and Black instead of the theoretical 50% obtained by these investigators. Wooley and Sebrell (25) have made essentially the same observations. It has been assumed, therefore, that racemization of tryptophane in the hydrolysates occurred to a comparable extent and the tryptophane in the bacterial cells has been corrected on this basis.

## DISCUSSION

It has been assumed previously that the proteins in a particular bacterial strain grown on media varying widely in composition have nearly constant proportions of their constituent amino acids. While some of the data reported by early workers (Table II) lends support to this view, much of the evidence is not highly accurate because inadequate methods were employed for the determination of amino

<sup>8</sup> Total nitrogen was determined by J. D. Murray.

TABLE I  
*Composition of Nutrient Media\**

Constituent	Medium A mg.	Medium B mg.	Medium C mg.
<i>dl</i> -Alanine	200	( 95)	
Asparagine, natural	200	( 298)	
<i>l</i> (+)-Arginine monohydrochloride	100	( 186)	
<i>l</i> (-)-Cystine	100	( 50)	
<i>l</i> (+)-Glutamic acid	200	(1100)	
Glycine	0	( 23)	
<i>l</i> (-)-Histidine monohydrochloride monohydrate	100	( 123)	
<i>l</i> (-)-Hydroxyproline	0	( 12)	
<i>dl</i> -Isoleucine	200	( 300)	
<i>l</i> (-)-Leucine	100	( 465)	
<i>dl</i> -Lysine monohydrochloride	100	( 312)	
<i>dl</i> -Methionine	100	( 143)	
<i>dl</i> -Phenylalanine	100	( 290)	
<i>l</i> (-)-Proline	0	( 435)	
<i>dl</i> -Serine	400	( 250)	
<i>dl</i> -Threonine	200	( 175)	
<i>l</i> (-)-Tryptophane	10	50	
<i>l</i> (-)-Tyrosine	50	( 268)	
<i>dl</i> -Valine	200	( 335)	
Casein†	0	5000	
Ammonium chloride	0	0	1000
Adenine sulfate	10	10	10
Guanine hydrochloride	10	10	10
Uracil	10	10	10
Dextrose	10,000	20,000	20,000
Sodium acetate	6,000	12,000	12,000
KH <sub>2</sub> PO <sub>4</sub>	500	500	500
K <sub>2</sub> HPO <sub>4</sub>	500	500	500
MgSO <sub>4</sub> ·7H <sub>2</sub> O	200	200	200
FeSO <sub>4</sub> ·7H <sub>2</sub> O	10	10	10
MnSO <sub>4</sub> ·4H <sub>2</sub> O	10	10	10
NaCl			1
Thiamine hydrochloride	0.5	0.5	0.5
Pyridoxine	0.8	0.8	0.8
<i>dl</i> -Calcium pantothenate	1	1	1
Riboflavin	1	1	1
Nicotinic acid	1	1	1
Biotin	0.003	0.003	0.003
p-Aminobenzoic acid	0.1	0.1	0.1
Folic acid‡	0.004	0.004	0.004

\* Per liter. The figures given in the parentheses are the calculated mg. of amino acids in 5 g. of casein.

† Acid hydrolysate employed.

‡ Product described previously (23).

TABLE II  
*Amino Acid Composition of Bacterial Protein\**  
 (Calculated to 16.0% nitrogen)

TABLE II (Continued)

Constituent	<i>Azotobacter</i>				<i>Lactobacillus</i>				<i>Escherichia coli</i>							
	<i>agilis</i>		<i>vinelandii</i>	<i>brevirostris</i>	<i>arabinosus</i>		<i>casei</i>		<i>pentosus</i>		<i>formicifaciens</i>					
	<i>L</i>	<i>M<sub>1</sub></i>	<i>M<sub>2</sub></i>	<i>N</i>	<i>O<sub>1</sub></i>	<i>O<sub>2</sub></i>	<i>P<sub>1</sub></i>	<i>P<sub>2</sub></i>	<i>Q<sub>1</sub></i>	<i>Q<sub>2</sub></i>	<i>R</i>	<i>S<sub>1</sub></i>	<i>S<sub>2</sub></i>	<i>T<sub>1</sub></i>	<i>T<sub>2</sub></i>	
Total nitrogen	9.95 9.5	9.00 8.3	13.2 7.3	3.95 10.1	10.38	10.08 3.3	7.98 3.6	7.49 3.1	10.22 3.1	9.74 4.8	13.98 4.8	13.38	13.08 7.8	10.28 7.8	9.96 6.6	10.28 6.7
Arginine														0.0	0.0	
Aspartic acid																
Cysteine	0.4	0.4		1.4	10.5	12.0		9.7	12.4	10.9	11.0					
Glutamic acid																
Glycine																
Histidine	0.1	0.1	7.9	0.1	1.9	1.7	2.0	1.9	1.6	1.5	2.4					
Isoleucine					5.5	5.6	6.7	6.2	5.1	4.9	7.0	6.3				
Leucine					6.2	5.9	6.8	6.8	5.2	5.1	7.5	7.5				
Lysine	5.8	4.1	2.5	3.6	5.9	5.2	7.7	4.9	4.6	6.9	6.3	5.9	10.5	7.4	4.8	
Methionine								1.1	1.1	1.1	1.0					
Phenylalanine								2.8	3.5	2.7	4.1					
Threonine								3.8	4.7	3.3	4.9					
Tryptophane	0.5	0.2	2.5	0.2	0.5	0.5	0.6	0.4	0.3	0.3	0.6	0.6	0.7	1.9	1.8	
Tyrosine					1.6	5.4	5.2	5.8	4.8	4.6	6.8	6.1	6.1	1.9	1.8	
Valine	2.1															

\* The figures refer to per cent of the protein.

† Assumed to be 10.7, the average of the other total nitrogen values listed.

## EXPLANATION OF TABLE II

A—Data reported by Tamura (4). *Mycobacterium tuberculosis (hominis)* was grown on a medium containing meat extract, sodium chloride, peptone and glycerine. The dried cells (yellowish-brown powder) were extracted repeatedly with ether and alcohol. The residual material was stirred with 5% sulfuric acid and, after the addition of water, a cell-free, amorphous precipitate resulted. The latter was extracted repeatedly with alcohol and ether and dried in *vacuo*. The dry product, containing 9.2% nitrogen, was hydrolyzed by refluxing it for 14 hours with 25% sulfuric acid. Arginine, histidine and lysine were determined by the Kossel and Kutscher method and phenylalanine, proline and valine were crystallized from the diamino acid filtrate.

B—Data reported by Johnson and Brown (9). *Mycobacterium tuberculosis (hominis and bovis)*, obtained from commercial sources, was extracted repeatedly with hot, anhydrous toluene and dried *in vacuo*. The residual material, containing 1.19% of P<sub>2</sub>O<sub>5</sub> and 10.2% of nitrogen, was employed for the determination of cystine, arginine, histidine and lysine by the Van Slyke nitrogen distribution method. The presence of tryptophane was indicated by a color test.

C—Data reported by Johnson and Coghill (10). *Mycobacterium tuberculosis* from a source different than that employed by Johnson and Brown (9) was defatted and analysed by the procedures described by Johnson and Brown (9). It has been assumed that these protein preparations had the same (10.2%) nitrogen content.

D—Data reported by Coghill (13). *Mycobacterium tuberculosis* (strain H37 obtained from the H. K. Mulford Company) was grown on a synthetic medium containing asparagine and ammonium chloride as the sole source of nitrogen. The cells were washed and dried *in vacuo*. The resulting nearly white powder was extracted repeatedly with cold ether. This product was extracted with cold water and the protein in the extract precipitated with acetic acid. The protein was purified by dissolving it in sodium hydroxide solution and reprecipitating with acetic acid. The precipitate was washed with acetic acid, alcohol and ether and was dried *in vacuo*. The nitrogen content of the final product was 12.6%. Arginine, histidine and lysine were determined by the Van Slyke nitrogen distribution procedure and tryptophane by a colorimetric method.

E—Data reported by Coghill (14). The previously described (13) defatted tubercle bacilli product was suspended for two weeks in a 5% sodium chloride solution. The supernatant fluid was centrifuged and dialyzed. The solution was saturated with ammonium sulfate and the precipitated protein dissolved in water and dialyzed. Alcohol was added to the solution and the precipitated protein washed and dried *in vacuo*. This product was redissolved in water and reprecipitated with alcohol. The final product, dried *in vacuo*, was a light gray powder. It contained 11.3% nitrogen. After further purification a nearly colorless powder containing 13.8% nitrogen was obtained. Arginine, histidine and lysine were determined by the Van Slyke distribution procedure and cystine, histidine, tryptophane and tyrosine by colorimetric methods.

F—Data reported by Campbell (11). *Mycobacterium tuberculosis (bovis)* was grown on broth. The dried cells were extracted with ethanol, toluene and petroleum ether. Histidine was determined colorimetrically in the phosphotungstic acid precipitate of the basic amino acids. Arginine was determined by the Van Slyke nitrogen distri-

bution method in the precipitate of silver arginate and silver histidinate. Lysine was determined from the total nitrogen in the arginine- and histidine-free filtrate. Glutamic acid was isolated as its hydrochloride and tyrosine was determined colorimetrically. The dry, defatted cells contained 10.6% nitrogen.

*G*—Data reported by Popper and Warkany (12). *Mycobacterium tuberculosis* was grown on bouillon and on a synthetic medium containing asparagine and ammonium lactate as the sole source of nitrogen. The cells were extracted with hot water and dried at 110°. The dried product was extracted repeatedly with ether. Tryptophane and tyrosine were determined colorimetrically.

*H<sub>1</sub>*—Data reported by Tamura (4). *Mycobacterium lacticola (perrugosum)* was grown on a medium of essentially the same composition as that described in section A. The *M. lacticola* cells were purified and analysed by methods analogous to those employed with tuberculosis cells. The protein material contained 8.2% nitrogen.

*H<sub>2</sub>*—Data reported by Tamura (5). *Mycobacterium lacticola (perrugosum)* was grown on a synthetic medium containing asparagine and ammonium lactate or ammonium carbonate as the sole source of nitrogen. The cells were purified and analysed by methods analogous to those previously described in section A. The protein material contained 9.63% nitrogen.

*I*—Data reported by Tamura (6). *Corynebacterium diphtheriae* was grown on a medium containing sheep kidney extract, peptone and sodium chloride. The cells were purified and analysed by methods analogous to those previously described (4, 5). The protein material contained 12.99% nitrogen.

*J<sub>1</sub>*—Data reported by Hetler (15). *Bacillus lactis aerogenes* was grown on a synthetic medium containing  $(\text{NH}_4)_2\text{HPO}_4$  as the sole source of nitrogen. The dried cells were extracted repeatedly with alcohol, ether and toluene. The defatted material was extracted with water, the residual material was extracted with saline and the final residue was extracted with 0.5% sodium hydroxide solution. The alkaline solution was acidified with acetic acid and the protein precipitate was washed with alcohol and ether and dried *in vacuo*. The final product contained 12.77% nitrogen. Arginine, histidine and lysine were determined by the Van Slyke nitrogen distribution procedure.

*J<sub>2</sub>*—Data reported by Hetler (15). *Bacillus lactis aerogenes* cells were grown, purified and analysed by the procedures described in section *J<sub>1</sub>*. The final product contained 14.51% nitrogen.

*K<sub>1</sub>*—Data reported by Omeliansky and Sieber (8). *Azotobacter chroococcum* was grown on a nitrogen-free synthetic medium. The cells were dried first *in vacuo* at 37° and then to constant weight at 100–105°. The final product contained 6.63% moisture, 4.16% ash and 2.07% nitrogen. Arginine, histidine and lysine were determined by the Van Slyke nitrogen distribution procedure.

*K<sub>2</sub>*—Data reported by Greene (17). *Azotobacter chroococcum* was grown on a nitrogen-free synthetic medium. The cells were suspended in acetone for several days and then dried in air. The final product contained 5.41% moisture, 4.00% ash and 4.00% nitrogen. Arginine, cystine, histidine and lysine were determined by the Van Slyke nitrogen distribution procedure. The methods by which tryptophane and tyrosine were determined were not given.

*L*—Data reported by Greene (17). *Azotobacter agilis* cells were prepared and the amino acids were determined by the methods described in section *K<sub>2</sub>*. The final product contained 8.55% moisture, 7.55% ash and 9.95% nitrogen.

*M<sub>1</sub>*—Data reported by Greene (17). *Azotobacter vinelandii* cells were prepared and the amino acids were determined by the methods described in section K<sub>2</sub>. The final product contained 9.06% moisture, 7.06% ash and 9.00% nitrogen.

*M<sub>2</sub>*—Data reported by Burris (18). *Azotobacter vinelandii* was grown on a nitrogen-free synthetic medium. The cells were transferred directly to 8 N sulfuric acid. Arginine was isolated as its flavianate, histidine as its mercury salt and monohydrochloride from the phosphotungstic acid precipitate of the diamino acids, and glutamic acid as its hydrochloride from the ethanol-insoluble calcium salts. Aspartic acid and lysine were determined from the nitrogen content of appropriate filtrates.

*N*—Data reported by Greene (17). *Azotobacter beijerinckii* cells were prepared and the amino acids were determined by the methods described in Section K<sub>2</sub>. The final product contained 5.61% moisture, 4.05% ash and 3.95% nitrogen.

*O<sub>1</sub>*—Data from this paper. The dry weight of *Lactobacillus arabinosus* cells grown on medium A (Table I) was 445 mg. The total nitrogen was 10.38 (10.31, 10.44)%. The values found for the glutamic acid isomers were *l*(+)-, 4.46%, and *d*(-)-, 2.38%.

*O<sub>2</sub>*—Data from this paper. The dry weight of *Lactobacillus arabinosus* cells grown on medium B (Table I) was 3.81 g. The total nitrogen was 10.08 (10.11, 10.06)%. The values found for the glutamic acid isomers were *l*(+)-, 3.87%, and *d*(-)-, 3.67%.

*P<sub>1</sub>*—Data from this paper. The dry weight of *Lactobacillus casei* cells grown on medium A was 108 mg. The total nitrogen was 7.98%.

*P<sub>2</sub>*—Data from this paper. The dry weight of *Lactobacillus casei* cells grown on medium B was 670 mg. The total nitrogen was 7.49 (7.48, 7.50)%. The values found for the glutamic acid isomers were *l*(+)-, 3.17%, and *d*(-)-, 1.37%.

*Q<sub>1</sub>*—Data from this paper. The dry weight of *Lactobacillus pentosus* cells grown on medium A was 510 mg. The total nitrogen was 10.22 (10.20, 10.24)%. The values found for the glutamic acid isomers were *l*(+)-, 3.53%, and *d*(-)-, 3.38%.

*Q<sub>2</sub>*—Data from this paper. The dry weight of *Lactobacillus pentosus* cells grown on medium B was 2.15 g. The total nitrogen was 9.74%. The values found for the glutamic acid isomers were *l*(+)-, 3.50%, and *d*(-)-, 3.13%.

*R*—Data from this paper. The dry weight of *Lactobacillus fermenti* cells grown on medium B was 3.00 g. The total nitrogen was 13.98? (14.02, 13.93)%. The values found for the glutamic acid isomers were *l*(+)-, 8.24%, and *d*(-)-, 1.33%. The writers' unpublished microbiological procedures were utilized for the determination of glycine, arginine, phenylalanine, cystine and methionine.

*S<sub>1</sub>*—Data from this paper. The dry weight of *Escherichia coli* (*communior*) cells grown on medium B was 530 mg. The total nitrogen was 13.38 (13.53, 13.24)%.

*S<sub>2</sub>*—Data from this paper. The dry weight of *Escherichia coli* (*communior*) cells grown on medium C was 190 mg. The total nitrogen was 13.08 (13.12, 13.02)%.

*T<sub>1</sub>*—Data reported by Eckstein and Soule (16). *Escherichia coli* (*Jordan*) was grown on a synthetic medium containing *l*(+)-alanine as the sole source of nitrogen. The cells were macerated with saline, the centrifugate was extracted repeatedly with ethanol and diethyl ether, and the residual material was dried *in vacuo*. The total nitrogen was 10.28%. Cystine, arginine, histidine and lysine were determined in the defatted material by the Van Slyke nitrogen distribution procedure. Tyrosine, tryptophane and cystine were determined by colorimetric procedures.

*T<sub>2</sub>*—Data reported by Eckstein and Soule (16). *Escherichia coli* (*Jordan*) was grown on a synthetic medium of the same composition as that referred to in *T<sub>1</sub>*.

excepting that *l*(-)-cystine was the sole source of nitrogen. The cells were purified and analysed by the methods referred to in T<sub>1</sub>. The total nitrogen was 9.96%.

T<sub>2</sub>—Data reported by Eckstein and Soule (16). The *Escherichia coli* cells were those described in T<sub>1</sub>. Arginine, histidine and lysine were determined by a modification of the Kossel procedure.

TABLE III

*Amino Acid Composition of Proteins from Three Plant and Animal Species*

Constituent	Grams amino acid/100 g. protein				Grams amino acid nitrogen/100 g. protein			
	Casein*	Skim milk powder†	Yeast‡	<i>Lactobacillus fermenti</i> §	Casein	Skim milk powder¶	Yeast¶	<i>Lactobacillus fermenti</i> ¶
Alanine	<u>1.9</u>				<u>0.30</u>			
Arginine	3.7	3.0	5.0	4.8	1.20	0.96	1.6	
Aspartic acid	6.1				0.64			
Cystine	0.3			0.09	0.04			0.01
Glutamic acid	22.5	21.5	14.7	11.0	2.14	2.04	1.4	1.05
Glycine	2.0	2.3		4.4	0.37	0.43		0.82
Histidine	2.9	2.7	2.6	2.4	0.79	0.73	0.71	0.65
Hydroxyproline	<u>0.2</u>				<u>0.03</u>			
Isoleucine	6.0	7.5	6.3	7.0	0.64	0.80	0.67	0.75
Leucine	9.3	10.2	7.2	7.5	0.99	1.09	0.77	0.80
Lysine	8.0	8.7	6.6	6.9	1.53	1.67	1.3	1.32
Methionine	2.9	2.3		1.3	0.27			0.25
Phenylalanine	5.8	5.1	3.9	4.1	0.49	0.43	0.33	
Proline	<u>8.7</u>				<u>1.05</u>			
Serine	5.0				0.67			
Threonine	3.5	(4.6)		4.9	0.41	(0.54)		
Tryptophane	1.2	(1.6)		0.6	0.16	(0.22)		0.08
Tyrosine	5.4				0.41			
Valine	6.7	7.4	7.1	6.8	0.80	0.89	0.91	0.81
Ammonia	1.6				1.32			
Total					14.25	9.80	6.09	6.54
Per cent of the total nitrogen					92	61	38	41

\* The data were taken from a paper by Dunn *et al* (23) excepting the values for lysine (20), histidine (1), and aspartic acid (26) and glycine (unpublished data from the authors' laboratory). The underlined values are considered to be the least reliable. The casein sample assayed by Dunn *et al* (23) contained 15.4% nitrogen corrected for moisture and ash.

† Unpublished data found by Dunn and coworkers from the assay of a spray-

acids. The present data, which are believed to be reasonably accurate, strongly support this hypothesis. It has been shown (Table II) that the amino acid composition of lactobacilli or *Escherichia coli* protein is nearly constant for defatted cells cultured on synthetic media of widely varying proportions of constituents.

There is also strong indication from the present data that total proteins in different strains of microorganisms do not differ markedly in amino acid composition. It may be noted from the data given in Table II that the percentage of any one of six amino acids in the proteins of four lactobacilli and *Escherichia coli* grown on three types of synthetic media does not differ by more than about 25% from the average percentage of the amino acid in the proteins of all of the strains of these microorganisms. It may possibly be true contrary to the views of Nencki (3), that total proteins, not only of bacteria but also of higher plant and animal forms, are similar in amino acid composition. The striking similarity in the amino acid composition of casein, skim milk powder, yeast protein and *Lactobacillus fermenti* protein shown in Table III appears to support this hypothesis. It may very well be true, however, that protein fractions prepared by extracting defatted cells with water, saline, sodium hydroxide solution, and other solvents may exhibit greatly different amino acid composition. Whether or not this assumption is correct can be determined only by analyses of higher accuracy than that inherent in the methods employed by early investigators who have presented evidence on this problem.

#### SUMMARY

*Escherichia coli* and four strains of lactobacilli have been grown on two types of synthetic culture media. The percentages of some amino

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dried skim milk powder containing 6.61% nitrogen corrected for moisture and ash. The values for threonine and tryptophane given in the parentheses were taken from Block and Bolling (27). All of the values are calculated to 16.0% nitrogen.

† Unpublished data found by Dunn and Merrifield from the assay of brewer's yeast, containing 8.55% total nitrogen uncorrected for moisture and ash. The values have been corrected to 16% total nitrogen. If corrected for purine nitrogen, the figures listed would be increased about 8%.

§ Data reported in this paper (Table II) for *L. fermenti* cells containing 13.98% nitrogen (fat and moisture free but uncorrected for ash). The values are calculated to 16% nitrogen.

|| The values are calculated to 15.4% nitrogen.

¶ The values are calculated to 16.0% nitrogen.

acids in the dried, defatted cells have been determined by micro-biological assay procedures. These data have been compared with the amino acid percentages of other microorganisms given in the literature. Some implications of these data have been presented.

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# The Biological Activity of O-Heterobiotin

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From the Nutrition Laboratories, Hoffmann-La Roche, Inc., Nutley, N. J.  
Publication No. 38

Received July 11, 1945

## INTRODUCTION

In a preliminary communication from this laboratory (1), the name "O-heterobiotin" was proposed for the analog of biotin wherein the hetero sulfur has been replaced by oxygen.<sup>1</sup> It was pointed out (1) that this substance shows remarkable growth-promoting activity for both *S. cerevisiae* 139 and *L. casei*. These observations warranted a further study of the biological activity of O-heterobiotin, the results of which are presented in this report.

The synthesis of this compound has also been reported by Hofmann (2), but without any statement of its activity. The published melting points and ultimate analyses from the two laboratories agree fairly well.

## EXPERIMENTAL

### Assay Methods

The activity of O-heterobiotin was explored for a number of biotin-requiring yeasts and bacteria in accordance with the following procedures.

*L. casei*—according to Shull, Hutchings and Peterson (3), as modified by Shull and Peterson (4). The acid produced was measured by electrometric titration to pH 5.6, the pH of the medium. The inoculated (blank) medium underwent little change in pH during the 3-day incubation.

*L. arabinosus* 17-5—according to Wright and Skeggs (5) except that the tubes were incubated at 37° rather than 30-33°, and titration was performed in the electrometric apparatus (to pH 6.8) rather than by colorimetric means.

<sup>1</sup> The O-heterobiotin used in the present study was synthesized in the Roche Laboratories by Dr. R. Duschinsky and L. A. Dolan (to be published).

*S. cerevisiae* <sup>2</sup> 139, 188, 4288, 4216. With these organisms, assays were carried out by one or more of the following procedures, with the stated modifications.

1. According to Snell, Eakin and Williams (6). The medium was seeded with 0.6 mg. of moist yeast cells per 100 ml., instead of 0.2 mg. The samples were placed in Evelyn colorimeter tubes, adjusted to a volume of 2.0 ml. and 10 ml. of the inoculated medium was added to each tube. The unplugged tubes were all slanted at the same angle in wooden blocks and incubated for 16 hours in a 30° forced-draft incubator room. After incubation, growth was stopped by adding 3 drops of 0.6% sodium azide solution.

2. According to Hertz (7). The medium was prepared as directed, placed in plugged flasks, autoclaved for 15 minutes at 15 lbs. and kept until used. The sterile medium was seeded with 0.6 mg. of moist yeast per 100 ml. The yeast was used directly from a 24-hour culture grown on Difco Wort agar. The samples were pipetted into Evelyn colorimeter tubes, diluted to 2 ml. and 10 ml. of the seeded medium was added to each tube. The unplugged tubes were slanted at the same angle in wooden blocks in a 30° forced-draft incubator room.

3. According to Atkin, Schultz, Williams and Frey (8). In view of the excellent results obtained in this laboratory with the "shaking" method of Atkin *et al.* for pyridoxine (9) and pantothenic acid (10), it was thought desirable to check the activity of O-heterobiotin by a similar shaking method.<sup>3</sup> The procedure is essentially the same as their published method for pyridoxine (9).

#### Activity Data

The preparation of O-heterobiotin used in the present work is optically inactive and represents one of the four structurally-possible racemates of 2'-keto-3,4-imidazolido-tetrahydrofuran-(2)-*n*-valeric acid. Hence, if it is assumed that only one enantiomorph is biologically active, all activity values given in this paper represent half the activity of this active form.

*Activity for L. casei.* The growth-promoting activity for *L. casei* deserves particular notice since O-heterobiotin is the only substance other than biotin which has been reported (1) to have any significant activity for this organism, with the possible exception of the diamino-carboxylic acid obtained from biotin by hydrolysis. For the latter, Dittmer and du Vigneaud (11) had reported an activity of less than 0.01%, while Stokes and Gunness (12) reported 7.2% for this compound obtained synthetically as the *d,l*-diamino acid sulfate, the latter value being based on the assumption that only the *d*-form is active.

<sup>2</sup> We are indebted to Dr. Vincent du Vigneaud of the Cornell University Medical School for a culture of *S. cerevisiae* 139. The other yeast cultures were obtained through the courtesy of Mr. Robert Light of the Fleischmann Laboratories.

<sup>3</sup> We are indebted to Drs. Charles N. Frey and Lawrence Atkin of the Fleischmann Laboratories for making the details of this method available to us prior to publication.

Representative growth curves of *d*-biotin and O-heterobiotin are given in Fig. 1, in a semi-logarithmic plot. The maximum growth response of O-heterobiotin is equal to that obtained with biotin, which indicates that the basal medium is adequate for the former. It is apparent, however, that the slopes of the two curves are dissimilar; therefore, when the various O-heterobiotin assay levels are calculated against the standard biotin curve, the results appear as drifting values. The average result of 8 assays calculated at the half-maximum point was 23% by weight corresponding to 22% on a molar basis.

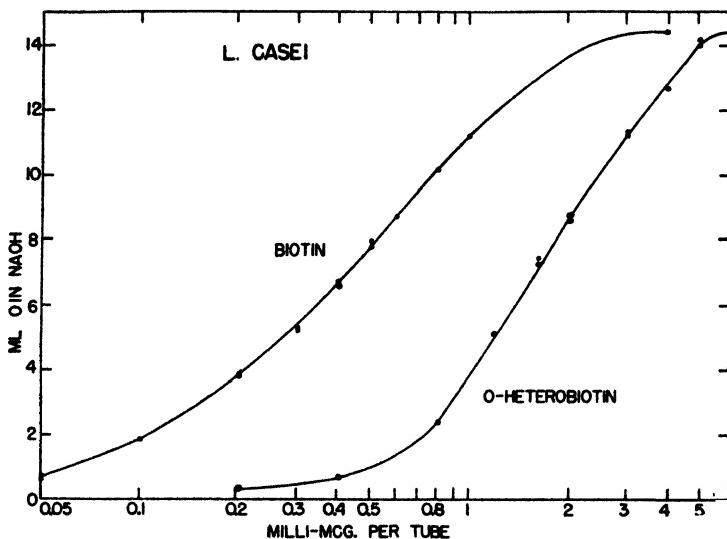


FIG. 1  
Typical *L. casei* Growth Curves (Semi-Logarithmic Plot)

*Inhibition of O-heterobiotin by desthiobiocytin.* An experiment patterned after that of Dittmer, Melville and du Vigneaud (13) on the inhibition of biotin by desthiobiocytin, as tested with *L. casei*, showed that O-heterobiotin, like biotin, is inhibited by desthiobiocytin. Fig. 2 gives the inhibition curve obtained by adding graded amounts of *d,l*-desthiobiocytin to tubes containing 1 m $\gamma$ <sup>4</sup> of O-heterobiotin. The molar inhibition ratio, M.I.R. (11), was calculated from the amount of desthiobiocytin required to depress the growth obtained with 1 m $\gamma$  of O-heterobiotin

<sup>4</sup> The symbol "m $\gamma$ " designates millimicrograms, cf. Snell, Eakin and Williams (6).

to that obtained with  $0.5 \text{ m}\gamma$ , based on a concurrent O-heterobiotin curve. It was not possible to run the O-heterobiotin inhibition in the same range as that used for biotin (11) because of the difference in activity levels. The M.I.R. so obtained is about 1200. *d*-Desthiobiotin has been reported to give a M.I.R. of 9100 with *d*-biotin (11), while *d,l*-desthiobiotin showed a value of 17,000 (17). The value of 1200 for O-heterobiotin, while based on a modified definition, indicates that desthiobiotin inhibits O-heterobiotin about as effectively as biotin.

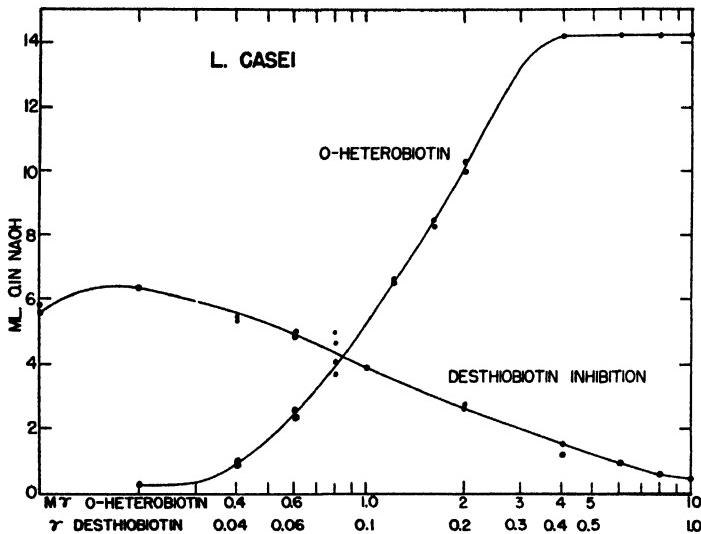


FIG. 2  
Effect of *d,l*-Desthiobiotin in Inhibiting the Growth Effect of  
O-Heterobiotin for *L. casei* (Semi-Logarithmic Plot).

*Activity of O-heterobiotin for L. arabinosus.* With this organism, O-heterobiotin displayed an average activity of 53% by weight, equivalent to 50% on a molar basis, when calculated at the actual assay levels. No drift was observed in any of the runs. Typical protocols are represented in Fig. 3. At the half-maximum,  $0.81 \text{ m}\gamma$  of O-heterobiotin per tube (10 ml.) is equivalent to  $0.43 \text{ m}\gamma$  of *d*-biotin.

*d,l*-Desthiobiotin, in amounts ranging from  $2.5$  to  $20\gamma$  per tube, exercised a progressive stimulatory action on  $1 \text{ m}\gamma$  of O-heterobiotin. This parallels the stimulatory effect of desthiobiotin on biotin with

*L. arabinosus*, described by Lilly and Leonian (16), which we have also been able to confirm.

*Activity of O-heterobiotin for yeast.* The activity of O-heterobiotin for yeasts was studied with *S. cerevisiae* 139, 188, 4288, 4216 and *Gebrüder Mayer*, and *S. carlsbergensis* 4228. Although different sensitivity ranges were observed for the various strains of *S. cerevisiae*, O-heterobiotin was found to be about 20% as active as *d*-biotin with all the methods and strains tested.

Typical activity curves for biotin and O-heterobiotin for *S. cerevisiae*

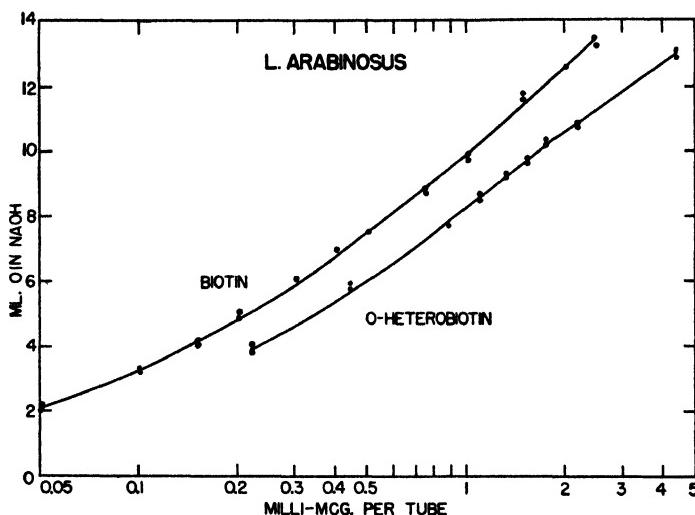


Fig. 3  
Typical *L. arabinosus* Growth Curves (Semi-Logarithmic Plot).

139 on the Hertz medium, are demonstrated in Fig. 4. Here, as in the *L. casei* assays, large drifts occur. The average result of six assays calculated at the half-maximum point is 22% on a molar basis. At this point an average amount of 0.70 mγ of O-heterobiotin is equivalent to 0.162 mγ of biotin.

Attempts to increase the activity of O-heterobiotin to the level of biotin for *S. cerevisiae* 139 by adding various possible stimulators were unsuccessful. Glutamic acid, aspartic acid, methionine, cysteine, cystine, tryptophane, guanine or uracil did not have the desired effect.

*S. carlsbergensis* 4228, a yeast which can synthesize biotin, grew

well on biotin-free medium. When biotin or O-heterobiotin was added to the medium, the growth was not changed.

It was found that dilute solutions of O-heterobiotin (containing about 0.1 m $\gamma$  per ml.) are not stable in the refrigerator for more than about two weeks. This suggested that the drifts might be due to partial decomposition of the compound in the assay tubes during sterilization and/or incubation. However, solutions which were sterilized by filtration through glass filters gave the same values as those sterilized by heating. Neither did any difference appear between assays which were inoculated the same day as set up or incubated 24

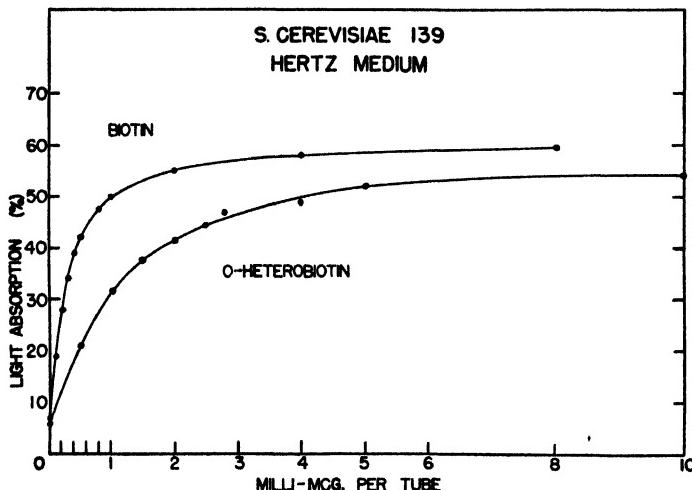


FIG. 4  
Typical Yeast Growth Curves

hours at 30°C. before inoculation. Recrystallized samples also showed the same drift.

The possibility that the drifting values might arise from regular variations in the rate of conversion of O-heterobiotin to biotin or a similar vitamer, was explored in runs with *L. casei* and *S. cerevisiae* in which the time of incubation was extended considerably beyond the usual periods. These rate experiments revealed no changes in the relative activities of biotin and O-heterobiotin, although the growth of the organisms increased progressively with increasing time of incubation.

*Combination with avidin.* From the definition of the avidin unit for biotin (15) and on the assumption that the combination of O-heterobiotin with avidin occurs in the same molecular ratio as with biotin, it may be estimated that 1.07 units of avidin would be required to inhibit 1 $\gamma$  of O-heterobiotin. Experimental confirmation of this estimate is provided in Table I, wherein it is shown that the requirement

TABLE I  
*Combination of Avidin with O-Heterobiotin*

O-heterobiotin m $\gamma$ per tube	Avidin* units per tube	Uncombined O-heterobiotin m $\gamma$	O-hetero- biotin inhibited m $\gamma$	Units of avidin required to in- activate 1 $\gamma$ of O-heterobiotin
	$\times 10^3$			
1.0	0.46	0.65	0.35	1.3
1.5	0.46	1.09	0.41	1.1
2.0	0.46	1.50	0.50	0.9

Method of Dittmer *et al.* (14) using Hertz medium.

\* This concentrate contained 2300 units per gram. 50 mg. were diluted in 2%  $(\text{NH}_4)_2\text{SO}_4$  to a concentration of  $0.46 \times 10^{-3}$  units per cc. One cc. of the final dilution was added to each tube.

approximates 1.1 units. In other experiments in which this stoichiometry was maintained, complete inactivation of O-heterobiotin occurred.

The fact that O-heterobiotin is avidin-combinable provides further support for du Vigneaud's theory (18) that the intact imidazolidone ring is required for combination with avidin.

*The mechanism of utilization of O-heterobiotin.* In view of the striking activity of O-heterobiotin for these bacteria and yeasts, it is interesting to consider the mechanism of utilization. In their work on the utilization of desthiobiotin by yeast, Dittmer, Melville and du Vigneaud (13) and Stokes and Gunness (12) have presented techniques for such studies, which we have drawn upon for use in the present work.

*A priori*, conversion of O-heterobiotin to biotin presents itself as a likely mechanism. Quantitative study of this mechanism calls for the analysis of mixtures of the two substances, which requires a differential method of assay. Because of the marked similarity, both qualitative and quantitative, in the activity of O-heterobiotin and biotin for the microorganisms studied, no such differential assay based on micro-

biological tests has come to hand yet. Nevertheless, it has been possible to exploit the existing differences in the extent and character of the responses of *S. cerevisiae* and *L. casei*, to elicit evidence bearing on the probability of such conversion.

Graded amounts of O-heterobiotin (Table II) were added to 40 cc. of the Snell medium, seeded with 2.4 mg. of *S. cerevisiae* 139 and

TABLE II  
*O-Heterobiotin Conversion Experiments*

O-heterobiotin added. <i>m<sub>γ</sub></i>	Apparent O-heterobiotin found in cells <i>m<sub>γ</sub></i>	Apparent O-heterobiotin found in medium <i>m<sub>γ</sub></i>	Apparent recovery of O-heterobiotin <i>per cent</i>
10	18	2	200
25	30	3	130
50	52	3	110
100	86	3	89

incubated for 16 hours at 30°. The cells were then separated from the medium by centrifugation. The medium was autoclaved without acid for 15 minutes, while the cells were autoclaved at 120° for 1 hour in 2N H<sub>2</sub>SO<sub>4</sub>. The solutions from the autoclaved cells were brought to pH 4, adjusted to volume and filtered. Both the autoclaved medium and the hydrolyzed cells were assayed with *S. cerevisiae* 139 in the Hertz medium. Biotin and O-heterobiotin standards were run concurrently.

As shown in Table II, practically all the added O-heterobiotin entered the cells. If the growth-promoting activity of the hydrolyzed cells is calculated as O-heterobiotin, as has been done in Table II, the apparent recoveries obtained range progressively from 200% at the lowest level of O-heterobiotin added to about 100% at the highest level. Hence conversion to a more active vitamer, presumably biotin, is demonstrated at the lower levels. Conversely, the growth may be calculated as biotin, in which case approximately 30% conversion is indicated at the lowest level, lesser conversion at the higher levels. These estimates seem reasonable because the two lower levels are below the level of maximum growth for the size of yeast inoculum employed, while the two higher levels are above.

*Influence of the source of sulfur on the utilization of O-heterobiotin.* In view of the qualitative demonstration of the conversion of O-hetero-

biotin to biotin or a biotin-like vitamer, it was of interest to consider the influence of different sources of sulfur on this transformation. For this purpose, the sulfate salts of the Snell, Eakin and Williams medium were replaced by equivalent chlorides. Growth of *S. cerevisiae* 139 was measured both in this and in the complete medium in the presence of

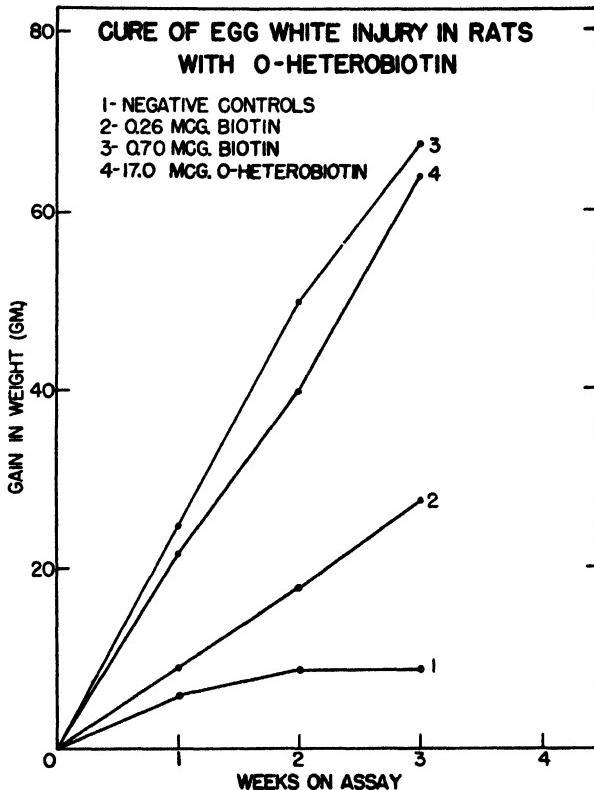


FIG. 5

either biotin or O-heterobiotin and the sulfur supplement. None of the compounds studied had any effect with either biotin or O-heterobiotin in the complete medium, with the exception that cysteine exerted a slight inhibitory effect.

In the sulfate-free medium, the sulfur compounds may be classified as follows according to the effects displayed: (1) sodium sulfate and

methionine supported good growth; (2) cystine, and possibly glutathione, supported slight growth; (3) sulfanilic acid and sulfasuxidine had no effect; (4) cysteine exerted a depressant effect. The results with sodium sulfate, methionine and cystine are similar to those found by Stokes and Gunness (12) in a similar experiment on the conversion of desthiobiotin to biotin.<sup>5</sup>

*The cure of egg white injury in rats with O-heterobiotin.* The procedure used in this laboratory for the animal assays has been described previously (17). In the present experiments, the diet contained 30% egg white and O-heterobiotin was administered by intraperitoneal injection. The activity of O-heterobiotin in curing egg white injury in rats under these conditions is 5% of the activity of *d*-biotin (Fig. 5). The significance of this value may best be judged from the fact that, of all the biotin derivatives which have been tested in the rat (14, 17, 19), this represents the only appreciable activity recorded to date.

## DISCUSSION

The qualitative and quantitative similarity between the biological behavior of O-heterobiotin and biotin permits classification of the former as a vitamer of biotin, as defined by Burk and Winzler (20). The properties of O-heterobiotin, however, do not correspond to those of the naturally-occurring, chemically-unidentified vitamers described by these authors, since their vitamers were either avidin-uncombinable or inactive for *S. cerevisiae* 139. These considerations do not eliminate the possibility that O-heterobiotin may occur in nature, as it may have been classified as biotin up to now on the basis of activity and combination with avidin in the usual methods of assay. The use of the rat assay and the development of more subtle microbiological methods of differentiation, cf. Leonian and Lilly (21), may provide the means of testing this possibility.

## SUMMARY

The growth-promoting activity of racemic O-heterobiotin for five strains of *S. cerevisiae* and for *L. casei* is about 25% of the activity of *d*-biotin, and for *L. arabinosus* 50% of *d*-biotin.

\* It is noteworthy that only slight growth of *S. cerevisiae* 139 occurs in the sulfate-free medium in the presence of biotin.

O-heterobiotin is inactivated by avidin in the same stoichiometric proportions as biotin. Desthiobiотin inhibits its growth effect for *L. casei*.

Experiments on the utilization of O-heterobiotin by yeast indicate that the mechanism involves conversion to biotin or a vitamer of similar activity. The influence of various sources of sulfur on this conversion is reported.

O-heterobiotin cures egg white injury in the rat at an activity level of 5% of *d*-biотin.

#### ADDENDUM

While the present paper was in press, a note by Pilgrim, Axelrod, Winnick and Hofmann appeared (*Science* 102, 35, 1945) on "The Microbiological Activity of an Oxygen Analog of Biotin." For *S. Cerevisiae* 139 and *L. arabinosus*, these authors used the same methods and reported essentially the same activities for their preparation of O-heterobiotin as are given above. However, using the Landy and Dicken medium for *L. casei*, they observed a substantially greater response, about 40% of *d*-biотin, than that reported here, *viz.* 22%. The explanation of this difference may well be the fact that less complete media, such as the Landy and Dicken, often induce greater growth of the vitamer relative to biotin, than do more complete media, such as that of Shull, Hutchings and Peterson.

A further note by Hofmann, McCoy, Felton, Axelrod and Pilgrim has also appeared recently (*Arch. Biochem.*, 7, 393, 1945), dealing with "The Biological Activity of Oxybiotin for the Rat and Chick." Their data show a biotin-like activity of about 5%, which is in good agreement with our findings in rats (*v.s.*).

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## Studies in Histochemistry .

### XVI. Methods for the Histochemical Localization of Adenosinetriphosphatase, Thiaminepyrophosphatase and Glycerophosphatase in Grains and Sprouts

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Received July 13, 1945

#### INTRODUCTION

The technique of Gomori (1) for the histochemical localization of acid glycerophosphatase in animal tissues has been varied in certain details by Wolf *et al.* (2) and Moog (3). In the present report, the adaptation of the technique to the demonstration of several phosphatases in grains and sprouts will be described. The Gomori method is based on the principle of precipitation *in situ* by lead of the phosphate liberated enzymatically in a microtome section of tissue, followed by conversion of the lead phosphate to the more easily visualized brown or black lead sulfide. To conserve substrate, particularly the adenosinetriphosphate, a hanging-drop technique was devised which requires only one drop of the substrate solution for each tissue section. Wheat was chosen as the test object for the present study and a preliminary report (4) of the portion of this work dealing with adenosinetriphosphatase has already appeared.

#### METHODS

##### *I. Preparation of Paraffin Sections*

###### *A. Kernel Sections*

1. Soak kernels in water for about 7 hours.
2. For longitudinal sections, cut off a layer from both the crease side and the opposite side of the kernel. For cross sections, cut off kernel just behind germ. This enables more efficient penetration of liquids.

3. Let kernels stand overnight in absolute alcohol. In the morning change to a mixture of 1 vol. absolute alcohol + 3 vols. *n*-butyl alcohol.

4. In the evening transfer to *n*-butyl alcohol.

5. The following morning place in xylol, and let stand until evening.

6. Transfer to a xylol-paraffin mixture containing just enough xylol to keep the paraffin in soln. at room temperature and let stand overnight. "Tissuemat" (Fisher Scientific Co., Pittsburgh) gave better results in our hands than paraffin and was substituted for the latter throughout. In a warm room the variety melting at 60–62° gave better sections than the material having a lower melting point.

7. Place in a soln. of 1 vol. xylol + 2 vol. melted paraffin in a 60° oven for 1–2 hours.

8. Infiltrate with melted paraffin for 2 hours in the oven, then change to fresh paraffin for 4 hours and finally embed.

9. Cut sections 10  $\mu$  thick and mount on slides with the aid of Mayer's albumin. (Combine 1 vol. of filtered fresh egg white with 1 vol. of glycerol and add a bit of camphor as a preservative.) Smear the liquid in a thin film on a slide with the finger, cover with water, transfer section to the slide, place in oven for 5 minutes at about 55° to soften paraffin and allow wrinkles to straighten out, drain off water with a towel, and allow to dry for 2 hours in the 55° oven. Store mounted sections in refrigerator until ready for use.

10. Remove paraffin from sections with 2 changes of xylol followed by 2 changes of absolute alcohol.

11. Dip slides into 0.5–1.0% collodion in alcohol-ether to cover section with a protective film; harden film by dipping into 80% alcohol, and wash with distilled water.

#### *B. Rootlet and Leaf Sections of the Sprout*

1. Place in the following solutions for 1 hour in each case, in the order given.

a. 70% alcohol

b. 80% alcohol

c. 65 cc. of 80% alcohol + 35 cc. of *n*-butyl alcohol

d. 45 cc. of 95% alcohol + 55 cc. of *n*-butyl alcohol

e. 25 cc. of absolute alcohol + 75 cc. of *n*-butyl alcohol

f. *n*-butyl alcohol

g. xylol

2. Follow step (6) under (A) in the preceding part, allowing the material to stand in the mixture for only 1/2 hour.

3. Subject the material to 3 changes of melted paraffin in the 60° oven during the course of 1 hour. If air bubbles are present in the leaves, apply suction to remove them.

4. Embed in paraffin colored red by stirring a few grains of Sudan IV in the molten material. In uncolored paraffin it is difficult to see the tissue in the sections.

5. Cut sections, mount on slides, remove paraffin, and protect with collodion film just as in steps 9, 10 and 11 under A.

## *II. Preparation of Frozen Sections*

*Kernel Sections* (rootlet and leaf sections of the sprout are too fragile to permit satisfactory frozen section technique).

1. Soak kernels 4–6 hours in water.

2. Mount in a drop of water on freezing head of microtome.

3. Cut sections 15  $\mu$  thick, keeping knife cold with dry ice, and transfer, with a needle cooled by dry ice, into 80% alcohol. (The 80% alcohol is used rather than water as, in the latter medium, the starchy endosperm disintegrates and separates from the rest of the section.)

4. Float the section onto a glass slide immediately. If wrinkled, straighten section in a drop of 70% alcohol.

5. Dehydrate by covering section with 5 successive drops of absolute alcohol, draining off after each drop is added.

6. Cover section with a small drop of 0.5–1.0% collodion soln. and harden film by dipping slide in 80% alcohol. Wash in distilled water.

## *III. Preparation of Substrate Media*

### *A. Glycerophosphatase Medium*

Combine the following ingredients, shake thoroughly, centrifuge, and use the clear liquid. The use of 0.01 M manganese sulfate and ascorbic acid as precipitate intensifier and enzyme activator (3) was omitted as good results were obtained without them.

a. 4 cc. 0.1 M acetate buffer of pH 5.1

b. 1 cc. 0.1 M lead nitrate

c. 0.6 cc. distilled water

d. 0.4 cc. 3.2% sodium glycerophosphate (because it was easily available, the mixture, containing 52%  $\alpha$  and 48%  $\beta$ , of the

Eastman Kodak Co. was used). Booth (5) showed that the  $\alpha$  compound is hydrolyzed more rapidly than the  $\beta$  by wheat phosphatase, and the  $\alpha$  has the additional advantage that its lead salt is more soluble than that of the  $\beta$  at this pH value (1).

### *B. Thiaminepyrophosphatase Medium*

Combine the following ingredients:

- a. 2 cc. 0.1*M* acetate buffer of pH 5.1
- b. 0.25 cc. 0.1*M* lead nitrate
- c. 0.25 cc. thiaminepyrophosphate (cocarboxylase) solution containing 10 mg.

### *C. Adenosinetriphosphatase Medium*

Prepare as follows:

- a. Dissolve 5 mg. of barium salt preparation of adenosinetriphosphate in 0.5 cc. 0.1*M* hydrochloric acid.
- b. Add 0.1 cc. of 1.11% sodium sulfate (amount required to precipitate the barium in the preparation used).
- c. Centrifuge and neutralize the supernatant with 0.1*M* sodium hydroxide using bromthymol blue as an outside indicator.
- d. To 0.25 cc. of this soln. add, in the following order, 0.75 cc. of acetic-lead soln. (2 vol. of 0.1*M* acetic acid + 1 vol. of 0.1*M* lead nitrate), 0.1 cc. of 0.1*M* calcium chloride, and 1.5 cc. of 0.1*M* sodium acetate. The acetate buffer in the mixture has a pH of 5.1.
- e. Centrifuge before use to remove the turbidity present.

### *IV. Preparation of Control Media*

Replace substrate solns. by distilled water in preceding media. The control media are identical for all three of the enzymes with the exception that, in the case of adenosinetriphosphatase, 0.1 cc. of 0.1*M* calcium chloride is added to 2.5 cc. of the soln.

### *V. Demonstration of Enzyme Activity*

1. Drain off excess water from section and cover with a small drop of substrate medium. Drain off the liquid and apply a second drop. Treat another section identically with control medium.
2. Place a hanging-drop slide over each slide bearing a section so that the drop is enclosed in the chamber formed by the depression.

The drop should not touch the chamber walls at any point. Invert the slides, leaving the section covered by the hanging drop. (For digestion periods of over 4 hours it is necessary to seal the edges of the two slides with vaseline or evaporation will become appreciable.) Since glycerophosphate is not particularly rare, a Coplin jar of this substrate medium may be employed instead of the hanging drop to simplify the technique.

3. Set in 37° incubator for digestion period shown in Table I.

TABLE I  
*Digestion Periods Required by Portions of Wheat Kernel and Sprout for Various Phosphatases*

Source of tissue	Enzyme		
	Glycero-phosphatase	Thiamine-pyrophosphatase	Adenosine-triphosphatase
Kernel, paraffin sections			
Embryo	1 hr.	1 hr.	2 hr.
Non-embryonic part	30 min.	30 min.	1 hr.
Kernel, frozen sections			
Embryo	15-30 min.	15-30 min.	15-30 min.
Non-embryonic part	5-10 min.	5-10 min.	5-10 min.
Rootlets, paraffin sections	8 hr.	2 hr.	3 hr.
Coleoptile and leaves, paraffin sections	24 hr.	20 hr.	24 hr.

4. After removing slides from incubator, wash sections with 3 changes of distilled water. (If vaseline was used to seal slides, remove with benzol.)

5. Dip into 2% acetic acid and wash well with distilled water.
6. Place in ammonium sulfide soln. (1 cc. of the compound to a Coplin jar of distilled water) for 2-3 minutes.
7. Wash with several changes of distilled water.
8. Dehydrate in 95% alcohol for 2-3 minutes followed by 5 minutes in absolute alcohol.
9. Clear in oil of thyme for 3-4 minutes and treat with 3 changes of xylol. (Treatment with xylol must be brief as the black precipitate indicating enzyme action is soluble to some degree in xylol.)
10. Mount in balsam.

#### DISCUSSION

The methods described are applicable to grains and sprouts other than those of wheat. However, the data on digestion periods in Table I

should not be taken too literally since species and varietal differences in enzyme activity may be expected, and further variations may result from differing conditions of growth, harvesting, and storage. In general it is necessary to determine the best digestion time for a given tissue by actual trial.

The results obtained by the application of these methods to kernels and sprouts of wheat will be given in a subsequent publication.

The thiaminepyrophosphate used was kindly supplied by Dr. H. G. Obermeyer of Merck and Co., Inc., Rahway, N. J., and the adenosinetriphosphate by Prof. M. B. Visscher, Dr. H. G. Wood, and Dr. M. F. Utter of the Physiology Department of the University of Minnesota Medical School.

#### SUMMARY

Details have been given for the adaptation of the Gomori technique to the histochemical localization of adenosinetriphosphatase, thiaminepyrophosphatase and glycerophosphatase in grains and sprouts, using wheat as the test object. A hanging-drop technique has been described which requires only one drop of substrate solution for each tissue section.

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# Influence of Methoxyl Content of Pectic Substances on the Action of Polygalacturonase

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Received July 17, 1945

## INTRODUCTION

Pectinase has been designated by Kertesz (1) as that group of pectic enzymes, mainly of microbial origin, which hydrolyze pectin and pectic acid into reducing sugars and other simpler substances. He designated the glycosidase responsible for the hydrolysis of polygalacturonic acid as polygalacturonase (Ehrlich's pectolase). The enzyme responsible for the hydrolysis of the methyl esters of pectin has been called pectase, pectin-methoxylase (1) and pectinesterase (2), the last name being preferable, as it clearly defines the action of the enzyme.

Little work has been done on the specificity of glycosidase hydrolysis of pectic substances, chiefly because of failure to obtain quantitative separation of glycosidase from other demonstrable pectic enzymes (*e.g.*, pectinesterase) in the available pectin enzyme preparations (3, 4). Mehlitz and Maass (5) found that during pectinase clarification of fruit juice, the decrease in viscosity not only parallels the decomposition of the pectin, as measured by the Ca-pectate method, but that it is also proportional to the demethylation of the pectin. Kertesz (1, 3), however, found that when pectin in solution is enzymically decomposed, the decrease in viscosity is greater proportionally than the increase in reducing value, and that the viscosity can be reduced by heat alone without any measurable change in reducing value or methoxyl content. He states that in the experiments reported by Mehlitz and Maass the pectinesterase action was only incidentally proportional to the action of the polygalacturonase of the complex. Waksman and Allen (6), working with a *Penicillium* enzyme, found that polygalacturonic acid was hydrolyzed about twice as fast as pectin, as measured by the increase in reducing value.

In the study reported here, the influence of methoxyl content (degree of esterification) of pectic substances on the action of poly-

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galacturonase was determined with preparations of this glycosidase which had been freed of pectinesterase by treatment with acid. Pectin, alkali-prepared and enzyme-prepared pectinic and pectic acids, and methylglycoside of polygalacturonic methyl ester were used as substrates. The initial rate, the course, and the extent of the reaction for each substrate were determined by measuring the increase in reducing value. Since little glycosidic hydrolysis was obtained without de-esterification of the pectin, the name polygalacturonase, suggested by Kertesz, seems well chosen.

### METHODS

*Pectinesterase Assay.* The method of assay, which consisted of measuring the rate of liberation of carboxyl groups at pH 4.0 in the presence of 0.05 M Ca<sup>++</sup> at 30°C. by titration with 0.02 N NaOII, was the same as that previously described (2, 7). As the reaction proceeded, calcium pectate gel formation was prevented by the presence of polygalacturonase. The results are expressed as specific activities, *i.e.*, milliequivalents of carboxyl groups liberated per minute per aliquot of enzyme, designated by the symbol [PE.u].

*Polygalacturonase Assay.* A modified Willstätter-Schudel hypoiodite method (8) was used to determine the increase in reducing value caused by the glycosidic hydrolysis of the pectic substances at 25°C. One ml. of enzyme of proper dilution was added to 99 ml. of solution of pectic substance at pH 4.0. The final concentration of substrate was 0.5%. Aliquots of 5 ml. were removed at definite time intervals and added to 0.9 ml. of 1 M Na<sub>2</sub>CO<sub>3</sub> in a glass-stoppered Erlenmeyer flask, followed by 5 ml. of standard 0.1 N iodine. After standing for exactly 20 minutes, the reaction mixture was acidified with 2 ml. of 2 M H<sub>2</sub>SO<sub>4</sub> and the residual iodine titrated with standard thiosulfate solution. The milliequivalents of reducing groups liberated were determined from a standard curve prepared from data obtained with glucose. The reducing value of galacturonic acid monohydrate, on an equivalent basis, was found to have coincided exactly with that of glucose. The conditions of the assay permitted the use of the linear portion of the standard curve. In this range 1 meq. of I<sub>2</sub> reduced corresponded to the liberation of 0.513 meq. of aldose. The activities are reported in milliequivalents of reducing groups liberated from pectic acid per minute per unit of enzyme, *i.e.*, [PG.u]<sub>ml.</sub>, [PG.u]<sub>mg.</sub>, and [PG.u]<sub>mg. PN.</sub> These signify the specific activity per ml., per mg. dry weight, or per mg. of protein nitrogen respectively. The ratios used throughout this paper are the quotients of the glycosidase specific activity divided by the esterase specific activity for the same aliquot of sample, *i.e.*, [PG.u]/[PE.u].

*Preparation of Pectinic Acids by Pectinesterase and Alkali.* All the preparations of pectinic and pectic acids were made from the same lot of 178 grade citrus pectin, so that the results would be strictly comparable.

The enzyme-prepared pectinic acids were obtained by adding 200 ml. of orange flavedo pectinesterase solution (7), containing 7.35 PE.u, to 100 g. of citrus pectin in 16 liters of solution at pH 7.5. The solution also contained 0.15 N NaCl. The

pH, as indicated by a glass electrode, was kept constant by the addition of 0.5 N NaOH. When an amount of alkali equivalent to the previously calculated amount of ester hydrolysis desired, had been added, an aliquot was removed, the pH of the aliquot adjusted to 1.5, and the pectinic acid recovered by precipitation and washing. An equal volume of acetone was used for precipitation of pectinic acids of methoxyl content above 5%. Acids of lower methoxyl content were precipitated as a result of pH adjustment. The pectinic acid was washed with dilute HCl at pH 1.5 and finally with acetone. All of the samples were dried in a vacuum oven at 65°C.

Alkali-prepared pectinic acids were obtained in a similar manner, with the exception that de-esterification was accomplished by keeping the reaction mixture at pH 10.25. Beckman's special glass electrode for alkaline solutions was used and the reaction was carried out at 25°C., in 0.15 M NaCl, which has been shown to catalyze the alkaline de-esterification of pectin (9, 10).

*Solubilization of Pectinic Acids.* Pectic acid and the low-methoxyl-content pectinic acids are insoluble in water as free acids. Furthermore, if the calculated amount of alkali for neutralization is added to a water suspension of pectic acid, the rate of solution is extremely slow. On the other hand, pectinic and pectic acids were easily and rapidly dissolved by keeping the pH of a water suspension at 4 with addition of alkali as solution took place. By this method 2% solutions of pectic acid were easily obtained.

*Zeisel Method.* Clark's (11) modification of the Viebock and Schwappach method for the determination of methoxyl groups was used. Errors due to retained alcohol (12) were avoided. The methoxyl contents of all samples were calculated on an ash- and moisture-free basis.

*Uronic Anhydride Determination.* A modified method of Lefèvre and Tollens (13), which consists of measuring the CO<sub>2</sub> evolved by decomposing the sample in boiling 12.5% HCl, was used to determine the uronic anhydride content of the various samples. Where the extent of hydrolysis is reported, the calculation is on the basis of uronic anhydride content, corrected for methoxyl, ash and moisture. All the pectinic and pectic acids used for these experiments had, on this basis, a uronic anhydride content of approximately 84%.

## RESULTS

*Action of Pectinase<sup>1</sup> on Pectin and Pectic Acid.* The rates of glycosidic hydrolysis of pectin and enzyme- and alkali-prepared pectic acids by pectinase were measured. In this case the alkali-prepared pectic acid was obtained by the hot alkali saponification procedure of Ahmann and Hooker (14), as recommended by a committee on saponification procedures for pectin (15). It can be seen from Fig. 1 that both pectic acid preparations were hydrolyzed at an identical rate, which was 17 times faster than the rate for pectin. This difference was surprisingly large, considering the results reported by Waksman and Allen (6).

<sup>1</sup> Pectinol 100 D, manufactured by Röhm and Haas Co.

Our results indicated that the rate of glycosidic hydrolysis of pectin might be dependent upon the pectinesterase content of the pectinase complex. To test this hypothesis it was necessary to remove the pectinesterase from the polygalacturonase.

*Removal of Pectinesterase from Polygalacturonase.* Only qualitative separations of pectinesterase from polygalacturonase in the pectinase complex have thus far been reported (3, 4). The glycosidase was found to be more resistant to inactivation by acid than was the esterase. By three subjections of the pectinase to pH 0.6 at 25°C. for 20 minutes,

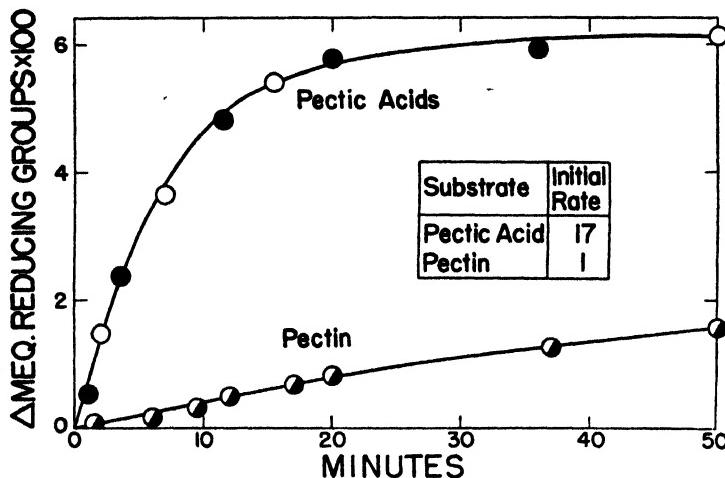


FIG. 1

Pectinase Hydrolysis of Pectin and of Alkali- and Enzyme-Prepared Pectic Acids.  
(Increase in milliequivalents of reducing groups per 5 ml. of  
a 0.5% solution of pectic substance.)

with readjustment to pH 4.0 and concentration of the material with  $(\text{NH}_4)_2\text{SO}_4$  between treatments, the esterase was decreased, on an equal-glycosidase basis, by more than 100 times. The results in Table I are typical. The final solution had 1350 times as much glycosidase as esterase. The same results were obtained with pectinase of an original ratio of 20. On occasion, four acid treatments were necessary to obtain a ratio of approximately 1000. For all practical purposes the final solution was free of pectinesterase. The polygalacturonase specific activity,  $[\text{PG.u}]_{\text{mg. PN}}$ , was increased from 0.17 in the original

pectinase to 0.30 in the case of the final material. No amylase could be detected in the polygalacturonase after the acid treatment, whereas the original material had, on a specific activity per gram basis, one-seventh as much amylase as polygalacturonase.<sup>2</sup>

It will be noted (Table I) that after the initial destruction of some polygalacturonase by acid, the subsequent treatments had little effect on the remainder, whereas the inactivation of the pectinesterase continued, although at a slower rate, with each subsequent treatment. This observation suggested that the acid treatment was affecting one of two polygalacturonases.

TABLE I  
*Removal of Pectinesterase from Polygalacturonase of Pectinase*

Number	Treatment	[PG.u] ml.	Per cent original PG.u	[PE.u] ml.	Per cent of original PE.u	Ratio <sup>1</sup>
1	None	0.167	100	0.0144	100	11.6
2	Acid treatment <sup>2</sup> of (1)	0.012	9.3	0.000086	0.8	140
3	10.6-fold concentrate <sup>3</sup> of (2)	0.116	8.5	0.00093	0.8	125
4	Acid treatment of (3)	0.076	7.7	0.00018	0.2	420
5	9.1-fold concentrate of (4)	0.68	7.6	0.0017	0.2	400
6	Acid treatment of (5)	0.42	6.9	0.00031	0.06	1350

<sup>1</sup> Ratio = [PG.u]<sub>ml.</sub>/[PE.u]<sub>ml.</sub>.

<sup>2</sup> The acid treatments consisted of adjusting the pH to 0.6 with 4 N HCl at 25°C. and after 20 minutes the pH was adjusted to 4.0 with 5 N NaOH.

<sup>3</sup> Concentration was effected by 0.85 saturation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at pH 4, filtration and solution of the precipitate in water.

This possibility was investigated by comparing the relative activities of pectinase and acid-treated polygalacturonase, of equal initial activities, acting on alkali-prepared pectic acid. The reaction courses, as opposed to those for  $\alpha$ - and  $\beta$ -amylases (16), were identical (Fig. 2). The extents of hydrolysis after 50 hours were 99 and 91%, respectively. The data were insufficient to show whether this difference in extent of hydrolysis was significant, although the difference was much greater than the experimental error of the reducing sugar determination. The

<sup>2</sup> The amylase assays were carried out at pH 4.9 with soluble starch as the substrate. The increase in reducing power was determined by the Willstätter-Schudel hypiodite method.

results suggest that if two polygalacturonases are present in the pectinase complex, they differ in their sensitivity to acid but not in their enzyme specificity.

*Action of Pectinesterase-Free Polygalacturonase on Pectin, Pectinic Acid and Pectic Acid.* The action of pectinesterase-free polygalacturonase on pectin, pectinic acids of various methoxyl content and pectic acid was measured. Fig. 3 shows the results obtained with enzyme-prepared and Fig. 4 those with alkali-prepared pectinic acids. In both figures the curve for the 9.47% methoxyl sample represents

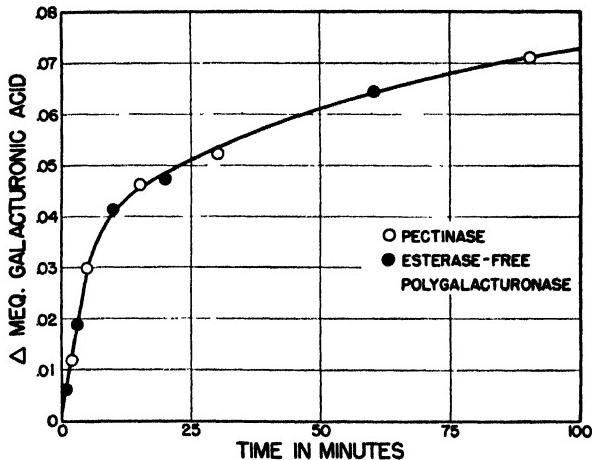


FIG. 2

Pectinase and Esterase-Free Polygalacturonase Glycosidic Hydrolysis of Alkali-Prepared Pectic Acid. (Increase in reducing groups calculated as galacturonic acid per 5 ml. of a 0.5% solution of pectic acid.)

the original pectin. All pectinic acid samples, regardless of the methoxyl content or method of preparation, had the same initial rate but the greater the methoxyl content the sooner the rate of glycosidic hydrolysis deviated from this initial linear rate. As may be seen from Figs. 3 and 4, the alkali-prepared pectinic acids of any given methoxyl content did not maintain the initial rate as long as the corresponding enzyme-prepared pectinic acids. This difference between the two series of pectinic acids will be discussed later. The data show that, with esterase-free polygalacturonase, rapid hydrolysis is obtained only with the de-esterified part of the molecule and that the rate on the fully

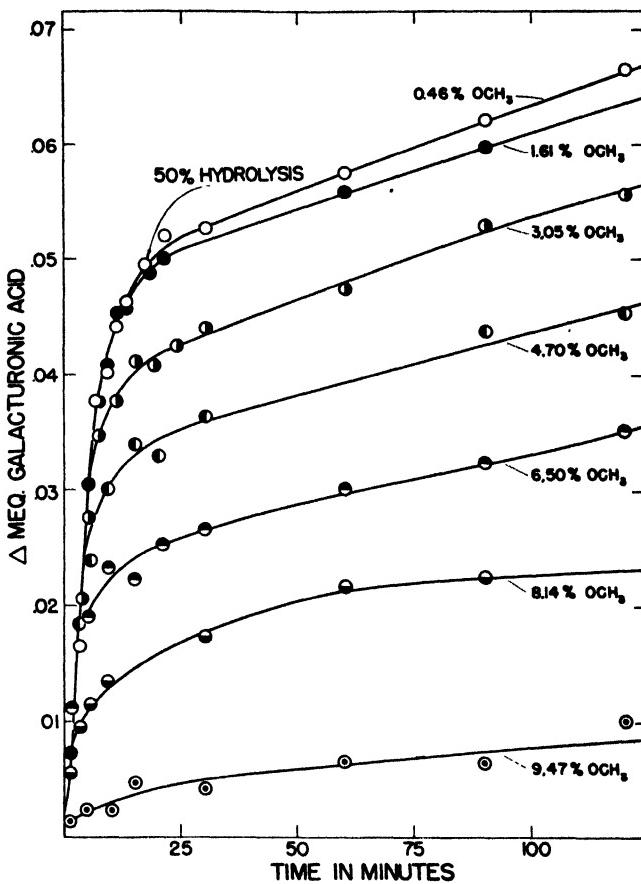


FIG. 3

Esterase-Free Polygalacturonase Hydrolysis of Pectic Substances. Methoxyl Content Adjusted by Orange Pectinesterase. (Increase in reducing groups calculated as galacturonic acid per 5 ml. of a 0.5% solution of pectic substance.)

esterified product can be, at a maximum, only 1/160th that of the de-esterified product. This difference is comparable to that obtained with certain proteinases acting on native and denatured globular proteins (17, 18). Hence the de-esterification of pectin makes the substrate available for glycosidase hydrolysis to the same magnitude that denaturation of proteins does for proteinases. It was to be ex-

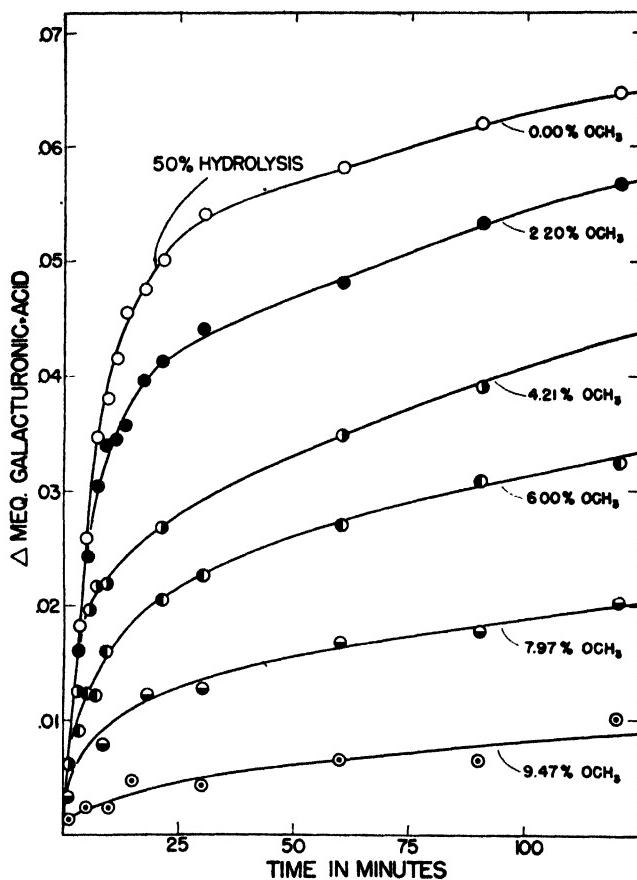


FIG. 4

Esterase-Free Polygalacturonase Hydrolysis of Pectic Substances. Methoxyl Content Adjusted by Alkaline Hydrolysis at pH 10.25. (Increase in reducing groups calculated as galacturonic acid per 5 ml. of a 0.5% solution of pectic substance.)

pected that a large initial rate for a very short time would be attained with pectin, since commercial pectins are not fully esterified. It is reasonable to conclude that the substrate for polygalacturonase is de-esterified pectin and that the action of commercial pectinase preparations is limited by their pectinesterase content.

*Action of Pectinesterase-Free Polygalacturonase on Fully Esterified Polygalacturonic Acid.* In order to demonstrate more conclusively that de-esterification of pectic substances must precede the glycosidic hydrolysis by polygalacturonase, the action of pectinesterase-free polygalacturonase was tried on a fully esterified polygalacturonic acid. The methylglycoside of polygalacturonic methyl ester of Morell, Baur and Link (19, 20) was prepared for this purpose. The action of esterase-free polygalacturonase on this substrate (0.22 PG.u added to 50 ml. of 0.5% solution at pH 4.0, both with and without 0.05 M lactate buffer) was only 1/2000th of that on pectic acid. After alkali de-esterification of this polygalacturonide, however, the polygalacturonase action (with 0.04 PG.u) was equal to that observed on pectic acid. Furthermore the de-esterified substrate after 50 hours was glycosidically hydrolyzed to more than 90% of the theoretical maximum. The extremely small rate observed with the fully esterified product might well have been due to the occurrence of a small amount of de-esterification, since 24–48 hour reaction periods were necessary before a significant increase in reducing value could be detected. Therefore, fully esterified pectic substances have little, if any, susceptibility to the action of polygalacturonase.

*Influence of Methoxyl Content of Pectic Substances on the Extent of Hydrolysis.* The reaction mixtures used to measure the initial reaction rates of the various pectinic acids were allowed to continue for 9 days to determine the effect of methoxyl content on the extent of hydrolysis. Actually, at 48 hours the hydrolysis differed from 9-day hydrolysis by only about 4%. In the case of the enzyme-prepared pectinic acids the extent of hydrolysis was an inverse linear function of the methoxyl content between 9.5 and 2.5% methoxyl and was complete only when the pectinic acids contained less than 2.5% methoxyl (Fig. 5). In the case of the alkali-prepared pectinic acids it was necessary to have hydrolyzed approximately 15% of the ester groups before any significant increase in the extent of glycosidic hydrolysis occurred, after which the extent became an inverse linear function of methoxyl content of the pectinic acids. Complete hydrolysis was obtained only after all of the ester groups had been removed.

*Influence of Methoxyl Content on Decrease in Viscosity During Hydrolysis.* In view of the conflicting reports of Kertesz (3) and of Mehlitz and Maass (5) concerning the viscosity decrease of pectin as correlated with glycosidic hydrolysis, the influence of methoxyl content

of the substrate on the viscosity change caused by esterase-free polygalacturonase was investigated.

For this purpose the viscosity was measured with an Ostwald pipet containing 10 ml. of a 0.5% solution of pectic substance and sufficient pectinesterase-free polygalacturonase to cause 1.7 % hydrolysis of pectic acid per minute. The viscosity

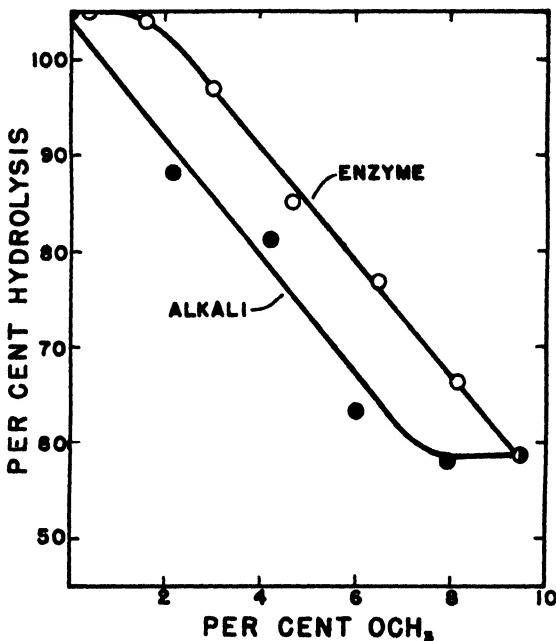


FIG. 5

Extent of Hydrolysis (9 days), on the Basis of Uronic Anhydride Content, of Enzyme- and Alkali-Prepared Pectinic and Pectic Acids Acted on by Esterase-Free Polygalacturonase.

changes were measured as a function of time, using the time for half drainage. The reaction was allowed to continue for 24 hours to obtain the maximum viscosity change, the final viscosity being only slightly higher than that of water.

With all the pectinic and pectic acids (Table II) 50% of the maximum viscosity change was found to have occurred, on the average, in 1.2 minutes, which corresponded to 2.0% hydrolysis. It will be noted (Figs. 3 and 4) that all of the pectinic acids were hydrolyzed at the same initial rate to well beyond the point of 2% hydrolysis. Such was

not the case with pectin, where approximately 6 times as long was needed to cause 2% hydrolysis, which corresponded to the time necessary for 50% of the change in viscosity. Hence most of the viscosity change caused by polygalacturonase occurred after more than 2% hydrolysis. However, the time necessary for 90% of the viscosity change (Table II) was a function of the degree of de-esterification, which was to be expected from the reaction course of the polygalacturonase.

TABLE II  
*Effect of Methoxyl Content on Viscosity Change Caused by Polygalacturonase<sup>1</sup>*

Substance <sup>2</sup>	Method of preparation	Per cent OCH <sub>3</sub>	50 per cent change	Time (min.) 90 per cent change
Pectin		9.47	7.6	>60
Pectinic acid	Enzymic	8.14	1.0	40
Pectinic acid	Enzymic	6.50	1.4	10
Pectinic acid	Enzymic	4.70	1.0	6.3
Pectinic acid	Enzymic	3.05	1.0	4.5
Pectinic acid	Enzymic	1.61	1.0	3.3
Pectinic acid	Enzymic	0.46	1.2	3.0
Pectinic acid	Alkaline	7.97	1.6	12
Pectinic acid	Alkaline	6.00	1.3	6.0
Pectinic acid	Alkaline	4.21	1.0	5.0
Pectinic acid	Alkaline	2.20	1.0	4.3
Pectic acid	Alkaline	0.00	1.4	4.0

<sup>1</sup> Sufficient pectinesterase-free polygalacturonase was used to cause 1.7% hydrolysis of pectic acid per minute.

<sup>2</sup> 10 ml. of a final concentration of 0.5% pectic substance was used.

Heat alone at the natural pH of pectin solutions causes a marked decrease in the viscosity of the solutions. According to the results of Kertesz (3) this viscosity change is not accompanied by a corresponding increase in glycosidic hydrolysis. However, in view of the small amount of hydrolysis necessary to cause a large change in viscosity, the effect of heat on the reducing value of pectin was reinvestigated. The results (Table III) show a definite glycosidic hydrolysis, the amount of which was approximately 2% after 1.5 hours, thus corresponding to approximately 50% of the viscosity change caused by heat (3). Hence, it appears that the viscosity change of pectins in acid solution, whether caused by heat or by polygalacturonase, is accompanied by hydrolysis. This alone does not mean that the glyco-

sidic hydrolysis is necessarily the cause of the viscosity change in the case of heat. However, Merrill and Weeks (21) have found that the energy of activation for the viscosity change caused by heat is about 30,000 cals., which corresponds to the energy necessary for the cleavage of a primary bond and, therefore, in view of the results above, it appears probable that heat changes the viscosity of pectin by virtue of glycosidic hydrolysis.

TABLE III  
*Effect of Heat on Reducing Value of Pectin<sup>1</sup> at pH 3.5*

Time of heating <sup>2</sup> (hrs.)	Meq. I <sub>2</sub> reduced	Meq. Galacturonic acid	Per cent hydrolysis
0	0.016	0.008	—
0	0.013	0.007	—
0	0.012	0.006	—
0	0.013	0.007	—
1.0	0.018	0.009	0.8
1.0	0.022	0.011	1.6
1.0	0.022	0.011	1.6
1.0	0.021	0.011	1.6
2.0	0.024	0.012	2.0
2.0	0.026	0.013	2.3
2.0	0.024	0.012	2.0
2.0	0.026	0.013	2.3
5.0	0.027	0.014	2.7
5.0	0.032	0.016	3.5
5.0	0.035	0.018	4.2
5.0	0.030	0.015	3.1

<sup>1</sup> Aliquots consisted of 4.92 g. samples of a 1.0% solution of pectinum N.F.

<sup>2</sup> Heated under reflux at 100°C.

**Reaction Kinetics.** In every case the course of hydrolysis of pectic acid by polygalacturonase goes through two stages: an initial rapid stage which proceeds to a point where approximately 45–50% of the glycosidic bonds are hydrolyzed and a second slow stage in which the residual bonds are hydrolyzed (Figs. 3 and 4). In a 0.5% solution the first stage is approximately 15 times more rapid than the second stage. These results are similar to those found by Myrbäck *et al.* (22, 23, 24) for the action of  $\alpha$ -amylase on starch, where the first 16% of the

glycosidic bonds were hydrolyzed at a rate 20 to 50 times faster than the second stage of hydrolysis.

The decrease in rate in the second stage of hydrolysis of pectic acid was not due to inhibition by reaction products as was shown by adding the "half-hydrolyzed" product (obtained by boiling a reaction mixture after 50% of the maximum hydrolysis had occurred) to a pectic acid solution to give a final concentration of 0.5% pectic acid and 0.5% "half-hydrolyzed" material. The initial rate of hydrolysis of this mixture, by a given amount of polygalacturonase, was equal to that obtained with pectic acid alone.

An attempt was made to determine the Michaelis-Menten dissociation constant,  $K_m$ , of the enzyme-substrate complex for both stages of the hydrolysis. It was impractical using the present method of analysis to measure dilute enough pectic acid solutions for the initial stage or concentrated enough solutions for the second stage. However, the  $K_m$  value for the first stage was less than 0.03% and, for the second stage, greater than 1.0%.

The kinetics suggest that after approximately 45–50% of the glycosidic bonds of pectic acid are hydrolyzed by polygalacturonase, the remaining material is a substrate which is attacked more slowly by virtue of its decreased affinity for the enzyme. This new substrate is possibly galacturonide.

#### DISCUSSION

It is evident that de-esterification must occur before polygalacturonase can act on pectic substances. Hence free carboxyl groups are necessary for action of polygalacturonase. Some indication of the amount of de-esterification necessary can be obtained from a comparison of alkali- and enzyme-prepared pectinic acids. Schultz *et al.* (25) found that pectinic acids of equal methoxyl content were weaker acids when prepared by citrus pectinesterase than by alkali or acid. The viscosity behavior of the former resembled that of pectic acid, while the latter resembled pectin. They offer the hypothesis, as suggested by Hills *et al.* (26), that pectinesterase de-esterifies portions of the galacturonide chain in a regular manner, while acid and alkali de-esterify at random. It might be hypothesized that polygalacturonase requires at least two adjacent free carboxyl groups in order to hydrolyze a glycosidic bond associated with these free carboxyl groups. Random de-esterification by alkali would, hence, have to proceed further before any appreciable increase in adjacent carboxyl groups

would appear. Since the value for the extent of hydrolysis of pectin lies on the linear portion of the curve for the enzyme-prepared pectinic acids (Fig. 5), it would appear that the presence of free carboxyl groups observed in citrus pectin may be attributed to pectinesterase action and not to the chemical treatment of processing.

The suggestion that the enzymic hydrolysis of pectic acid to galacturonic acid goes through a digalacturonide stage is not without precedent in the enzymic hydrolysis of polysaccharides; for example, amylases produce mainly maltoses from starch and cellulase hydrolyzes cellulose to cellobiose. Attempts will be made to find other enzymes which cause a rapid hydrolysis of the postulated digalacturonide.

Morell, Baur, and Link (20) concluded that the methyl glycoside polygalacturonic methyl ester they obtained was 8 to 10 galacturonic acid units in size. The observation that after de-esterification this material was hydrolyzed by polygalacturonase at a rate equal to that observed on pectic acid does not necessarily mean that the enzyme attacks the two substrates, so largely different in molecular size, at identical rates. Analysis of the fully esterified product, with precautions taken to avoid errors due to retained alcohol (12), indicates that the product described by Morell, Baur, and Link is 2 to 3 times larger than they suggest.<sup>3</sup>

#### SUMMARY

1. Pectinase complex hydrolyzed pectic acid, prepared with either pectinesterase or alkali, at an initial rate 17 times faster than it hydrolyzed pectin.
2. The polygalacturonase of the pectinase complex was essentially freed from pectinesterase by several acid treatments (pH 0.6), concentrating with  $(\text{NH}_4)_2\text{SO}_4$  between treatments. By this procedure the ratio of polygalacturonase to pectinesterase, on a specific activity basis, was changed from 10 to 1350. After this treatment the polygalacturonase followed the same reaction course when acting on pectic acid as did the pectinase, thus showing that the treatment did not alter the enzymic specificity.

3. Only the de-esterified portions of pectic substances were found to be susceptible to the action of polygalacturonase. This requirement was demonstrated by measurements of initial rates, courses and

<sup>3</sup> A detailed report of these observations will be made elsewhere.

extents of hydrolysis caused by pectinesterase-free polygalacturonase acting (a) on the methyl glycoside of polygalacturonic methyl ester (fully esterified pectic substance), (b) on pectin and (c) on pectinic and pectic acids prepared with alkali and with orange pectinesterase. The rate of hydrolysis for pectic acid was 160 times that for pectin containing 9.5% methoxyl. Initial rates of glycosidic hydrolysis on all pectinic acids containing 8.1% methoxyl and less were identical; however, the greater the methoxyl content the sooner the rate departed from the initial linear rate. For alkali-prepared pectinic acids the rate departed sooner than for corresponding enzyme-prepared products. The hydrolysis of the methyl glycoside of polygalacturonic methyl ester proceeded at a rate 1/2000th of that observed after de-esterification. The latter rate was the same as that observed with pectic acid.

4. Extent of hydrolysis of pectic substances was a function of the degree of de-esterification. For a given methoxyl content the extent was greater for enzyme- then for alkali-prepared pectinic acids. This observation and others suggest that at least two adjacent free carboxyl groups are necessary for polygalacturonase action.

5. Enzymic hydrolysis of 2% of the glycoside bonds of any of the substrates caused 50% of the total viscosity change. Heat, which causes a decrease in the viscosity of pectin, was shown to produce a corresponding glycosidic hydrolysis of pectin at pH 3.5.

6. The hydrolysis of pectic acid by polygalacturonase goes through two stages: an initial rapid stage which extends up to approximately 45–50% hydrolysis of the glycoside bonds, followed by a slow stage in which the residual bonds are hydrolyzed. The first stage was 15 times as rapid as the second. The “half-hydrolyzed” material was not inhibitory to the hydrolysis of pectic acid. The Michaelis-Menten dissociation constant,  $K_m$ , for the first stage was less than 0.03% and for the second, greater than 1.0%. These observations suggest that the second stage was the hydrolysis of digalacturonic acid.

We are indebted to Rosie Jang and Katherine Denman for technical assistance and to Margaret Losce for the preparation of graphs.

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# Simultaneous Actions of Polygalacturonase and Pectinesterase on Pectin

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Received July 17, 1945

## INTRODUCTION

The necessity for de-esterification of pectic substances before polygalacturonase can act on them was shown in a previous paper and, further, the glycosidic hydrolysis of pectin by the pectinase complex was found to be limited by its pectinesterase content (1). It was also previously demonstrated (2) that, by proper control of the concentration of cations, at least 50% of the maximum activity of pectinesterase can be obtained at all pH values from 4 to 9, thus permitting the use of this enzyme at the acid pH values necessary for polygalacturonase action. The simultaneous actions of polygalacturonase and pectinesterase on pectin and the effect of fortification of the pectinase complex with both crude and purified pectinesterase obtained from orange flavedo have now been investigated.

## METHODS

The methods of enzyme assays used were the same as those previously described (1, 2). The polygalacturonase reaction mixtures acting on 0.5% pectin solutions were buffered with 0.05 M lactate buffer at pH 4.0 and were 0.1 M with respect to  $Mg^{++}$ . Magnesium ion in this concentration caused 48% inhibition of polygalacturonase acting on pectic acid, whereas the lactate buffer was without effect. The ratios used throughout are the quotients of the polygalacturonase specific activities divided by the pectinesterase specific activities for the same reaction mixtures,  $[PG.u]/[PE.u]$ , as previously defined (1). The pectinesterase activities were the initial activities obtained at pH 4.0, with 0.10 M  $Mg^{++}$  used for activation, acting on 20 ml. of 0.5% pectin solution. The rates of action of the polygalacturonase in mixtures of various enzyme ratios, acting on pectin, were compared with the optimum rate, i.e., the rate of the polygalacturonase acting on pectic acid under the reaction

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conditions described above. For any particular experiment a constant amount of polygalacturonase was added and the ratio varied by the addition of various amounts of pectinesterase. Citrus pectin (178 grade), as previously described (1), was used as pectin substrate.

## RESULTS

*Effect of Polygalacturonase on the Action of Pectinesterase.* In order to ascertain the effect of polygalacturonase on the activity of orange

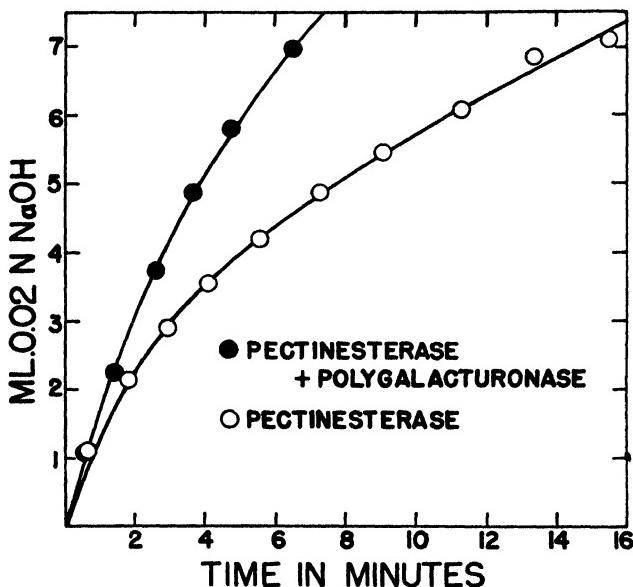


FIG. 1

The Effect of Polygalacturonase on Pectinesterase Activity Under Approximately Optimum Reaction Conditions for the Former. 0.04 PE.u was used per 20 ml. of reaction mixture and where the effect of polygalacturonase was determined, 0.4 PG.u was added per 20 ml. of reaction mixture.

flavedo pectinesterase, when the two enzymes were acting simultaneously, the activity of the esterase was determined both with and without glycosidase at pH 4. The results (Fig. 1), with 0.04 PE.u, show that polygalacturonase had a favorable effect on the esterase activity, in that a higher rate was maintained longer. This effect might well have been due to the removal, by virtue of glycosidic hydrolysis, of inhibition by the pectic acid formed, which has been

shown to inhibit pectinesterase action (3). It is evident that pectinesterases of plant origin can be used in conjunction with polygalacturonase.

*Enhancement of Polygalacturonase Action on Pectin by Pectinesterase.* Pectinesterase-free polygalacturonase (1) was used for these experiments. The ratios, which were varied from 750 to 0.05, were adjusted with either crude or purified dialyzed pectinesterase obtained from orange flavedo (2). The amount of polygalacturonase added per 100

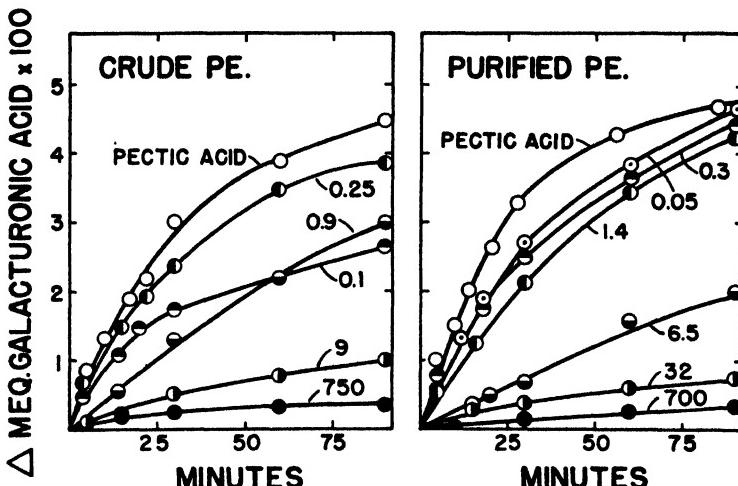


FIG. 2

The Effect of Added Orange Flavedo Pectinesterase on the Glycosidic Hydrolysis of Pectin by Polygalacturonase. With crude pectinesterase 0.019 PG.u per 100 ml. was used and 0.025 PG.u in the case of the purified esterase. The numbers represent the  $[PG.u]/[PE.u]$  ratios in respective reaction mixtures. The pectic acid curve represents the optimum rate obtainable.

ml. of reaction mixture was equivalent to 0.019 PG.u, where crude pectinesterase was used, and 0.025 PG.u in the case of the purified pectinesterase. The specific activities of the pectinesterase were  $[PE.u]_{mg. PN} = 0.15$  and  $[PE.u]_{mg. PN} = 3.0$ , respectively, for the crude and purified enzyme. Where crude enzyme was added to the polygalacturonase (Fig. 2), the activity of the polygalacturonase on pectin was increased with increasing additions of pectinesterase until, at a ratio of 0.25, the initial rate was approximately that of pectic acid. However, smaller ratios (more pectinesterase) decreased the

activity of the polygalacturonase. The decrease was probably due to inert protein in the crude pectinesterase, since the addition of inert protein (crystalline  $\beta$ -lactoglobulin) to pectic acid inhibited the action of polygalacturonase. On the other hand, when adjustments were made with purified pectinesterase (Fig. 2), even a ratio of 0.05 caused little, if any, inhibition. The maximum activity observed by the addition of the purified esterase approached that of pectic acid. Therefore, the addition of pectinesterase to polygalacturonase caused,

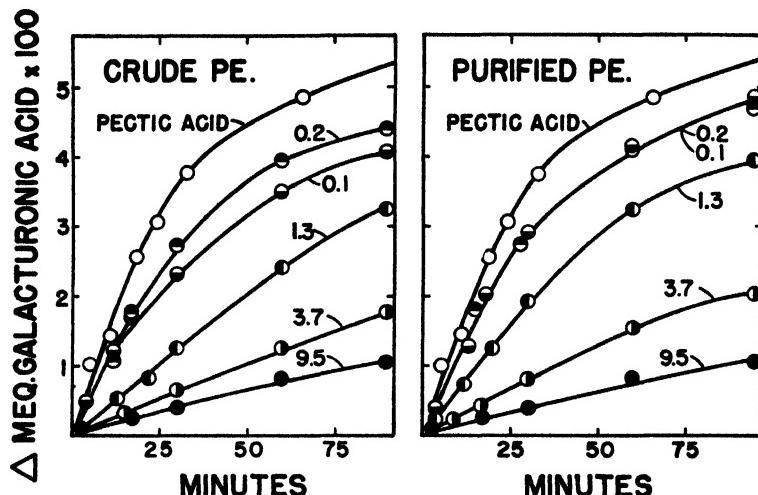


FIG. 3

The Effect of Added Orange Flavored Pectinesterase on the Glycosidic Hydrolysis of Pectin by the Pectinase Complex. 0.028 PG.u was used per 100 ml. of reaction mixture. The numbers represent the [PG.u]/[PE.u] ratios in the respective reaction mixtures. The pectic acid curve represents the optimum rate obtainable.

at a maximum, an increase of 60 times the glycosidic hydrolysis of pectin over that observed in its absence.

*Fortification of Pectinase Complex with Pectinesterase.* Both crude and purified pectinesterase were used to fortify commercial pectinase complex<sup>1</sup> having a ratio of 9.5 under the conditions described above. The initial rate of glycosidic hydrolysis by the pectinase complex (Fig. 3) acting on pectic acid (PG.u = 0.028) was 11 times greater

<sup>1</sup> Pectinol 100 D, manufactured by Röhm and Haas Co.

than on pectin. The addition of crude pectinesterase in increasing amounts up to a ratio of 0.2 caused increasing glycosidic hydrolysis. As before, the addition of crude esterase to give ratios less than 0.2 caused inhibition. On the other hand, purified esterase in large amounts caused little, if any, inhibition. The maximum increase in the rate of glycosidic hydrolysis caused by the addition of pectinesterase (ratio of 0.2 or less) to the pectinase complex was 73% that of pectic acid. This corresponds to an increase in efficiency of glycosidic hydrolysis of pectin by pectinase complex of more than 7 times. It is thus apparent that pectinesterase of plant origin can be used to fortify the fungal pectinase complex.

### DISCUSSION

Pectinesterase of plant origin acting on pectin simultaneously with either polygalacturonase or the pectinase complex can cause an increase in the glycosidic hydrolysis so that the rate approaches that observed on pectic acid. In order to use orange flavedo pectinesterase under the assay conditions used for polygalacturonase (pH 4.0), 0.10 M Mg<sup>++</sup> is necessary for activation (2). This concentration of Mg<sup>++</sup> caused 48% inhibition of the action of polygalacturonase, probably by combination with the substrate. Varying the Mg<sup>++</sup> at a constant addition of pectinesterase would vary the enhancement of polygalacturonase, to the extent that the Mg<sup>++</sup> affected the pectinesterase (2) as well as affecting the polygalacturonase. Other plant esterases, *e.g.*, pectinesterase from tomatoes, are active at acid pH values in lower salt concentrations (unpublished results). From the present study it follows that the commercial use of the pectinase complex as a means of removing pectin as an undesirable mixture constituent may be improved by supplementing the usual enzyme mixture with pectinesterase of plant origin.

### SUMMARY

1. Polygalacturonase, acting simultaneously with pectinesterase on pectin at pH 4.0, had a favorable effect on the rate of de-esterification and on maintenance of the rate, as compared with action of pectinesterase alone. This favorable effect was probably due to the removal, by glycosidic hydrolysis, of pectic acid, which inhibits pectinesterase.
2. In sufficiently high concentrations pectinesterase enhanced the

action of polygalacturonase on pectin to an extent that approached the rate observed on pectic acid. Higher concentrations of crude pectinesterase were inhibitory, probably because of the inert protein accompanying the esterase. No inhibition was observed with higher concentrations of purified pectinesterase.

3. Pectinesterase of plant origin can be used to fortify the pectinase complex.

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# The Induction of Dormancy in Vegetative Yeast Cells by Fat and Carbohydrate Storage and the Conditions for Reactivation \*

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Received July 11, 1945

## INTRODUCTION

It has long been known that yeasts store both fats and carbohydrates and the principal conditions controlling the storage of these reserve materials have been fairly well worked out. It was not known, however, that cells containing abundant accumulations of reserve materials are in a state of dormancy. They are unable to take up O<sub>2</sub>, to give off CO<sub>2</sub>, or to bud, either in a phosphate buffer solution or in a buffer-glucose solution in the Warburg apparatus. The cells begin to respire, ferment and bud after a few hours exposure to a medium containing a full supply of soluble vitamins, nitrogenous nutrients and sugar. Neither sugar alone nor vitamins alone can break dormancy. Dormancy is broken when the yeast cell is brought into a complete nutrient. The mechanism has a high survival value, as it prevents the cell from "wasting" its reserves, for it can only begin to grow under conditions in which continued or considerable growth is possible.

Meissner (1900) studied the appearance and disappearance of glycogen in the yeast cell and showed that cells filled with glycogen produced more than the theoretical amount of CO<sub>2</sub> in fermenting a sugar substrate. He also found that glycogen accumulates in the cell and attains a maximum at the end of the principal fermentation when it begins to disappear from the cell even before all the sugar is consumed. He called the CO<sub>2</sub> produced from glycogen, the *respiration-CO<sub>2</sub>*, and that produced by fermentation of external substrate, the *fermentation-CO<sub>2</sub>*. He designated glycogen as a temporary reserve used by the cell through an endogeneous diastatic enzyme. He pointed out the survival value of this mechanism and drew attention to Sachs' (1897) concept that the deposition of an insoluble carbohydrate inside a semi-permeable membrane enables the cell to take in soluble carbohydrate continuously by osmotic pressure.

\* This work was supported by a grant from Anheuser-Busch, Inc., St. Louis.

Wager and Peniston (1910) studied the same question with cytological techniques. They found that glycogen was deposited in the cell in the form of small granules which coalesced to form a solid mass of glycogen which almost completely filled the cell. This reserve was then metabolized and (in beer wort) a second or third storage of glycogen occurred, followed in each case by metabolism of the stored carbohydrate.

Smedley-Maclean and Hoffert (1923, 1924) and McAnally and Smedley-Maclean (1935) showed that both carbohydrates and fats accumulated in the cell as a result of continued feeding with sugars. They did not express their analyses in conventional percentages, but by disregarding the ash and taking certain liberties with their figures, one may conclude that an original culture containing approximately 59% protein, 36% carbohydrate and 5% fat, could be altered by continued feeding to produce a yeast containing about 20% protein, 46% carbohydrate and 34% fat. They found that phosphates increased the deposition of both reserves and that maltose seemed to increase the carbohydrate reserve. In addition they pointed out that an excess of oxygen favored storage of fat.

Henneberg (1926) also pointed out that the presence of phosphates favored the deposition of glycogen, while chlorides seemed to inhibit its deposition. He found that the maximal protein content of the cell was 67% and he was able to obtain yeasts containing only 22% protein. He stated that the amount of protein in a cell stands in inverse relation to the amount of glycogen (and presumably fat). He pointed out that the yeast cells collected directly from fruits are generally rich in glycogen and, furthermore, that yeasts kept in moist condition on filter paper live longer if they contain large amounts of glycogen. Some apiculate and lactose-fermenting yeasts were unable to store glycogen.

#### *Cytological Observations on Carbohydrate and Fat Storage*

The vegetative budding yeast cell, in the logarithmic growth phase contains a very large, centrally located vacuole and stains a light golden yellow with iodine (Fig. 1). It contains a few tiny fat globules. If these cells are placed under conditions of relatively low oxygen tension and supplied with an abundance of sugar, after budding has ceased, granular deposits of glycogen appear in the cytoplasm, which finally deform the vacuole and diminish its size (Figs. 3, 4, 5 and 6).

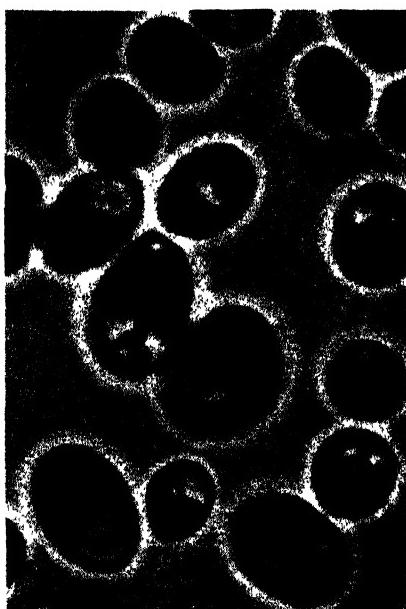
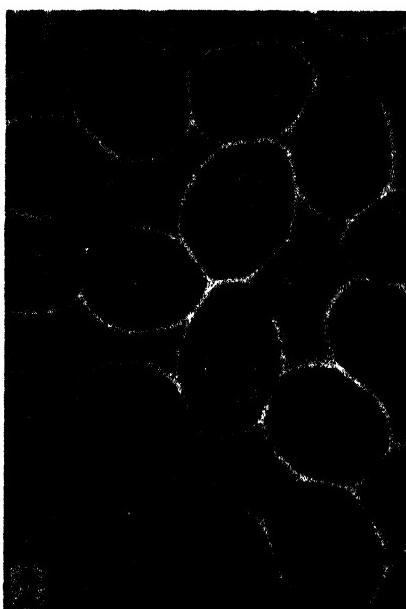
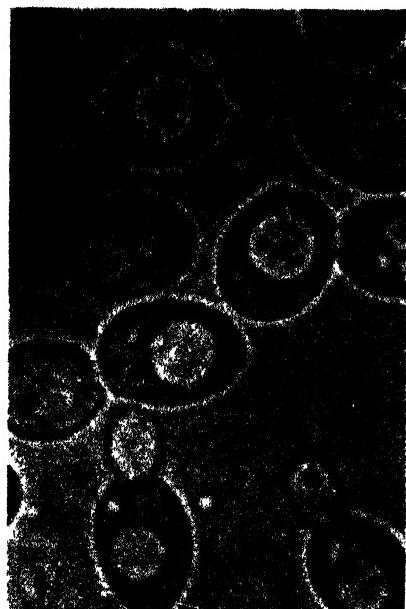
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FIG. 1. Vegetative Budding Cells in Logarithmic Growth Phase with Large Spherical Vacuoles.—These cells are glycogen-free and stain golden yellow with iodine. They usually contain about 25% carbohydrate.

FIG. 2. Budding Cells in the Lag Phase.—The vacuole appears multiple, but is actually single, each lobe being connected to the main body. The vacuole is deformed, probably by some reserve substances which have not been completely metabolized.

FIG. 3. Unstained Cells Containing a Heavy Glycogen Deposit.—The vacuole is crowded to one end of the cell and surrounded by fat globules, and the cytoplasm is highly refractive.

FIG. 4. Same as Fig. 3.—Stained with iodine showing the accumulation of granular glycogen and revealing the position of the vacuole.



If the cells are well-aerated and supplied with an abundance of sugar, fat globules appear and increase in size and number and also tend finally to obscure the vacuole, although it retains its spherical form (Fig. 7); non-granular carbohydrate also accumulates in the cytoplasm.

#### *Granular Glycogen*

The granular carbohydrate reserve in yeasts which stain dark reddish brown with Lugol's iodine-potassium iodide solution has been called "glycogen" by the early workers and I shall use this term in spite of the fact that recent chemical studies suggest that the substance may not be identical with liver glycogen (Cori, personal communication). If the yeast is merely suspended in a drop of this stain, the cells usually shrink considerably and, if they contain glycogen, the entire contents of the cell are stained a dark reddish brown. There is a distinct advantage in suspending the yeast cells in .01% methylene blue under a cover slip and allowing a small drop of Lugol's at one edge of the wet mount to diffuse slowly through the fluid. Observations are made at points intermediate between the densely stained and unstained regions in order to observe the effect of different concentrations of the stain. In a cell which, if overstained, would appear solid reddish brown, one may observe 20 or more small isolated granules. Occasionally these granules are linked by connecting bands. Hundreds of small glycogen granules can be seen occasionally in cells in addition to 15 or 20 larger ones. These observations suggest that, in most of the cells containing a dark-staining glycogen, the distribution is similarly non-homogeneous with the basic granular structure obscured by overstaining.

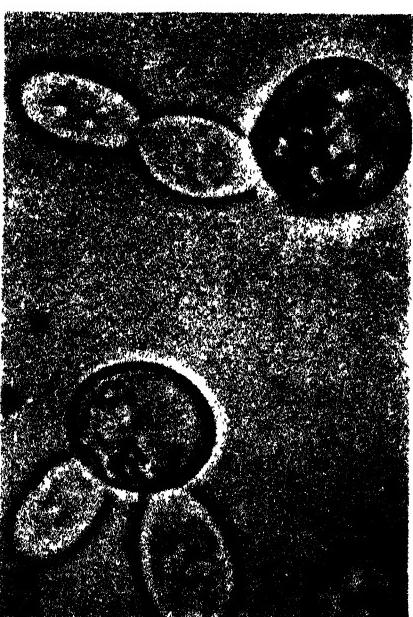
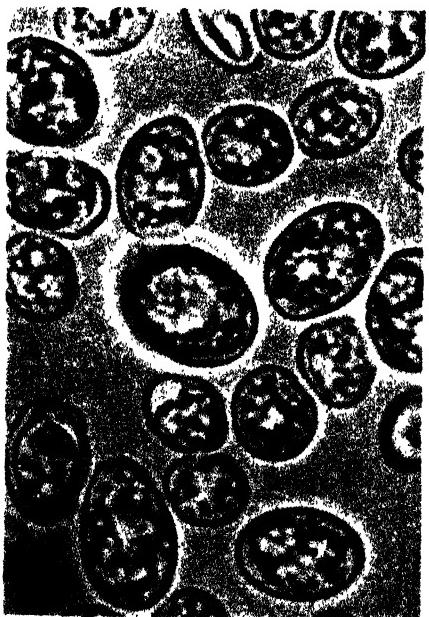
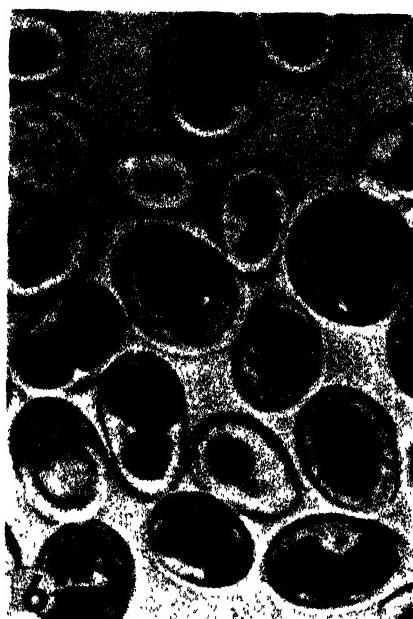
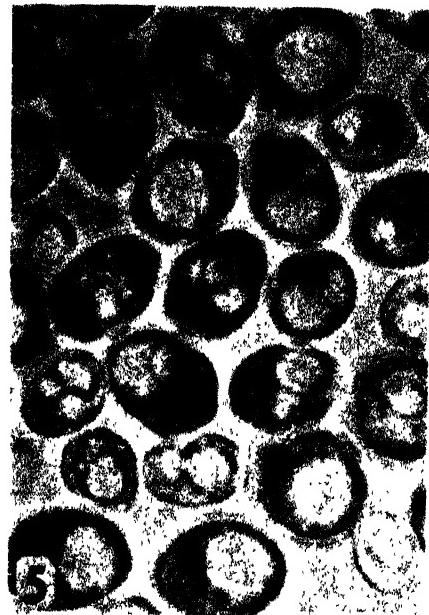
Unstained cells containing glycogen can be recognized by the high refractive index of the cytoplasm (Fig. 3). The nuclear vacuole in a glycogen-containing cell is often concealed by the glycogen. The visible vacuoles often appear to be multiple (Figs. 4 and 5), but critical observation shows that the small vacuoles are all inter-connected with each other by fine canals and are merely separate compartments of one major vacuole. This is consistent with the view

FIG. 5. An Early Stage of Glycogen Deposition.—Shows the multiple vacuole.

FIG. 6. Deposition of Glycogen in High Sugar Concentrations, Showing Very Dense, Usually Bipolar, Granules.

FIG. 7. Cells Filled with Fat Granules.—Produced by continued additions of sugar to aerated nutrient broth.

FIG. 8. Germination of Dormant Cells Loaded with Fat,



already expressed (Lindegren, 1945) that each yeast cell contains only a single vacuole. In many of the glycogen-containing unstained cells in which no vacuole is visible, staining with Lugol's solution often reveals the vacuole compressed into the middle of the cell by a surrounding sheath of glycogen. The vacuole in a glycogen-containing cell is usually very small.

Budding is retarded or inhibited in cells containing much glycogen, and only occurs after the glycogen has begun to disappear from the cell. Growing cells, during the lag phase (Fig. 2), contain enough unidentified reserve to obscure or deform the vacuole. After the cell has completed one or two divisions, the refractive index drops and the vacuole reappears or loses its deformity.

Deposition of granular glycogen is irregular in high (12%) concentrations of sugar and rarely fills the whole cell (Fig. 6). The stained granules are darker than in lower concentrations of sugar. Many small granules may coalesce, often forming two large polar deposits. Small granules of glycogen may be linked by ares of glycogen. At lower sugar concentrations (4%) the deposition of glycogen continues until it fills the entire cell with the exception of a small region at the end into which the vacuole is crowded, or the vacuole may be concealed in the center of the cell inside the spherical envelope of glycogen (Figs. 3, 4 and 5). After the deposit has reached a maximum, the glycogen disappears on aeration, by peripheral disintegration or solution, with a decrease in the density of the glycogen mass. Finally a single large diffuse granule is found in the cell, and the vacuole has regained its original size. Deposition of glycogen does not ordinarily begin until at least half the total number of cells that are to be formed are present. Under favorable conditions, involving low oxygen tension and continued addition of sugar, all the cells may become filled with glycogen.

Stainable glycogen does not accumulate in well-aerated yeast cultures grown in 1% glucose broth. The nuclear vacuole attains its maximal size under these conditions.

Glycogen is never found in the vacuole of the living cells but some dead cells contain glycogen in the vacuole. Most of the dead cells remaining in a culture which has been reactivated after glycogen deposition contain glycogen in the cytoplasm. Either glycogen tends to deposit in dead cells, or dead cells are unable to metabolize their accumulation of glycogen.

*Non-granular Carbohydrate*

Dark brown (granular) glycogen is deposited rather regularly in deep broth cultures in test tubes. Glycogen-free cells (which usually contain about 20% of an unspecified carbohydrate) grown in aerated 1% glucose broth stain golden yellow with iodine (Fig. 1). Cells from well aerated cultures which have received additional sugar differ from both the above by staining with iodine without any dark brown granular deposit. The entire cytoplasm is light brown, suggesting general distribution of a non-granular carbohydrate throughout the cell. The vacuoles are round, centrally located and usually large, indicating that the non-granular carbohydrate does not deform the vacuole, at least in the early stages of its deposition.

*Fat*

Fat is also stored in yeast if the culture is well aerated and well supplied with phosphate and sugar. Fat first appears as an accumulation of highly refractive droplets around the vacuole. Deposits are often polar or comprise a network of streptococcal-like threads of granules which sometimes branch and are closely appressed to the surface of the vacuole. In most types of *S. cerevisiae* the fat globules tend to increase in numbers and to enlarge individually as the culture becomes older and storage increases. The addition of alcohol or the application of heat causes many of the fat globules to coalesce. Dead cells usually contain coalesced fat globules, just as they often contain glycogen. Fat invariably accumulates in yeast cells in shallow Erlenmeyer cultures.

*Dormancy of Yeast Cells Grown on a Rich Natural Medium*

A medium favorable for sporulation was developed in this laboratory (Lindegren and Lindegren, 1944) containing extracts from beet, apricot, grape and yeast. After a culture has grown for several weeks on slants of this rich natural medium, the cells contain abundant deposits of fat and, although all the cells are alive, they are dormant. Subsequent experiments were undertaken to reproduce dormancy by loading the cells with reserve materials in broth cultures, but we were unable to obtain cells with precisely the appearance of those taken from pre-sporulation agar, which appears to be much more heavily packed with larger fat granules.

Yeast cells collected from fruits in nature also contain heavy deposits of reserve materials. Rich natural media may contain accessory substances that promote and stabilize the formation of reserve materials.

#### *Activation of Dormant Cells*

A diploid culture ( $155 \times$  EmlA) was grown 10 days on an agar slant of pre-sporulation medium. Practically all the cells were full of fat (Fig. 11). The vacuoles were clearly outlined in the cells loaded with fat, indicating that fat and glycogen deposition occurs without deformation of the vacuole and that the cytoplasm becomes relatively rigid. The cells were transferred to 6% glucose nutrient broth on the shaker and examined 1 hour later. Much of the fat had disappeared from half of the cells (Fig. 12). Iodine-potassium iodide showed a deposit of non-granular carbohydrate. At the second hour, most of the cells were budded and half of the budded cells showed a few fat granules. The vacuoles appeared to be multiple and obscured in the growing cells. In 3 hours, there was very little visible fat left in the cells, nearly all of which contained multiple or obscured vacuoles. Practically all the cells had budded, showing that the culture was viable.

A Warburg analysis was made on the cells taken directly from the pre-sporulation slant and shaken with phosphate buffer containing 4% dextrose, but with no other nutrients. The Q values after 150 minutes were  $Q_{O_2} = -25$ ;  $Q_{CO_2}^O = -41$ , and  $Q_{CO_2}^N = 0$ . During the same period, all the cells in the Erlenmeyer containing nutrient broth had budded. Only 2% of the cells from the Warburg vessel had budded; 98% of the cells showed little or no change. Although these cells were unable to ferment anaerobically, they were not completely dormant, as was indicated by their absorption of oxygen.

This experiment showed that dormant cells which were almost

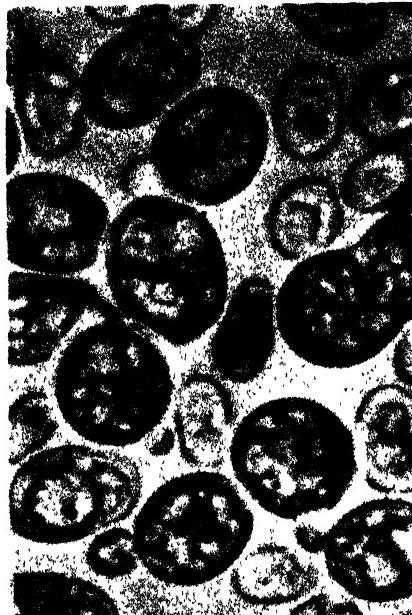
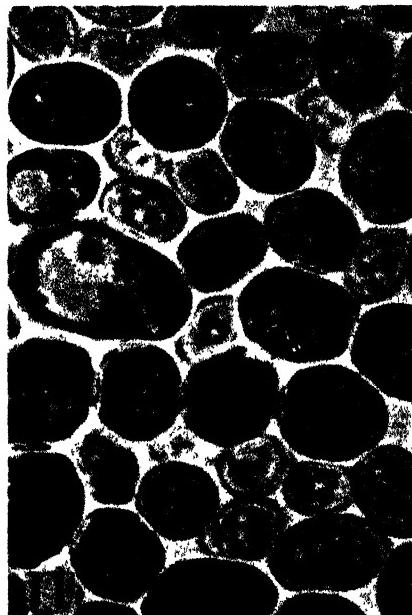
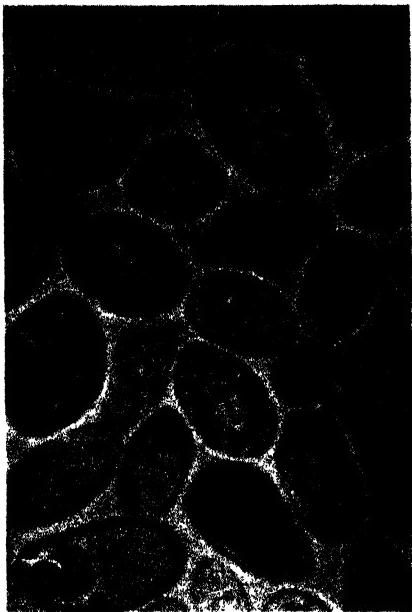
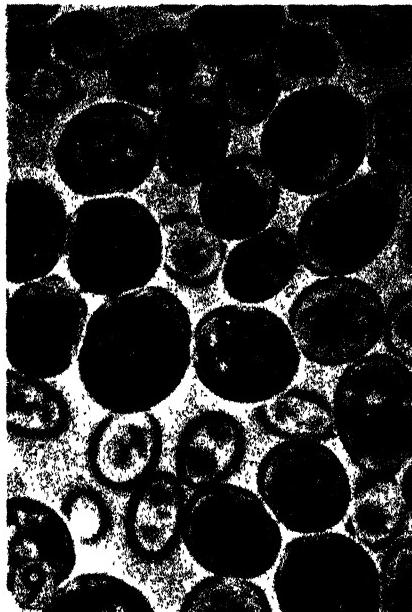
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FIG. 9. Cells from Pre-sporulation Agar at an Early Phase.—Many contain only glycogen. Few of the cells have attained true dormancy.

FIG. 10. A Later Phase of Development on Pre-sporulation Agar. —Some of the cells are sporulating and the reserves in others are breaking down preparatory to sporulation.

FIG. 11. Cells from a Pre-sporulation Slant Which Are Nearly All Dormant.—Each one contains large reserves of fat and glycogen, obscuring the vacuole.

FIG. 12. Germination of Dormant Cells.—Like those shown in Fig. 11, revealing the vacuole breaking through its network of chains of fat globules.



completely inactive in a phosphate buffer and dextrose, regained their respiratory activity in a few hours in a nutrient medium.

Cells of a standard baking yeast, strain A, which had been grown on pre-sporulation agar, were collected from the agar surface and washed with  $M/15\text{ KH}_2\text{PO}_4$ . Three Warburg vessels were inoculated with equal amounts of dormant cells. Each vessel contained 4% of glucose in solution. The first received phosphate buffer, the second received 1% of corn-steep-water solids, and the third received .3% ammonium sulfate, biotin (2  $\gamma$  per liter) and pantothenic acid (200  $\gamma$  per liter).

We have reported previously that *S. cerevisiae* is incapable of synthesizing biotin and pantothenic acid (Lindegren and Lindegren, 1945), and these substances were added, together with ammonia, to see if they would break the dormancy of the fat-filled yeast cells. The evolution of  $\text{CO}_2$  in mm.<sup>3</sup> in the presence of  $\text{O}_2$  was measured and is plotted and shown in Fig. 13. The cells suspended in sugar were dormant but dormancy was broken in both the other vessels, the action being much more rapid in the rich nutrients.

This experiment cannot always be duplicated, because cells from the pre-sporulation agar slants are not always in precisely the same condition. They may be either in the early stages of growth preliminary to fat storage (Fig. 9) or have gone beyond that point to the early stages of sporulation (Fig. 10).

#### *Storage of Reserve Materials in Broth Cultures*

A study was made of the conditions controlling the storage of reserve materials in the cell in an attempt to develop a reproducible method for getting larger amounts of dormant cells than one can collect from a pre-sporulation agar slant.

The effects of low and moderate aeration and different concentrations of sugar on glycogen deposition were studied by comparing growth in 50 cc. nutrient broth in a 500 cc. Erlenmeyer and with growth in 30 cc. broth in an 8  $\times$  1 inch test tube, using the following percentage concentrations of sugar: 12, 8, 4, 2 and 1. Each culture was observed daily to determine the percentage of dead cells and the percentage containing glycogen. The final dry weight of yeast in mgm./cc. was measured. Table I shows that (1) in low concentrations of sugar, glycogen disappears much more rapidly than in high, (2) the cells

die much more rapidly in high than in low concentrations of sugar, and (3) the efficiency of converting sugar to yeast is much higher in low sugar concentrations than in high.

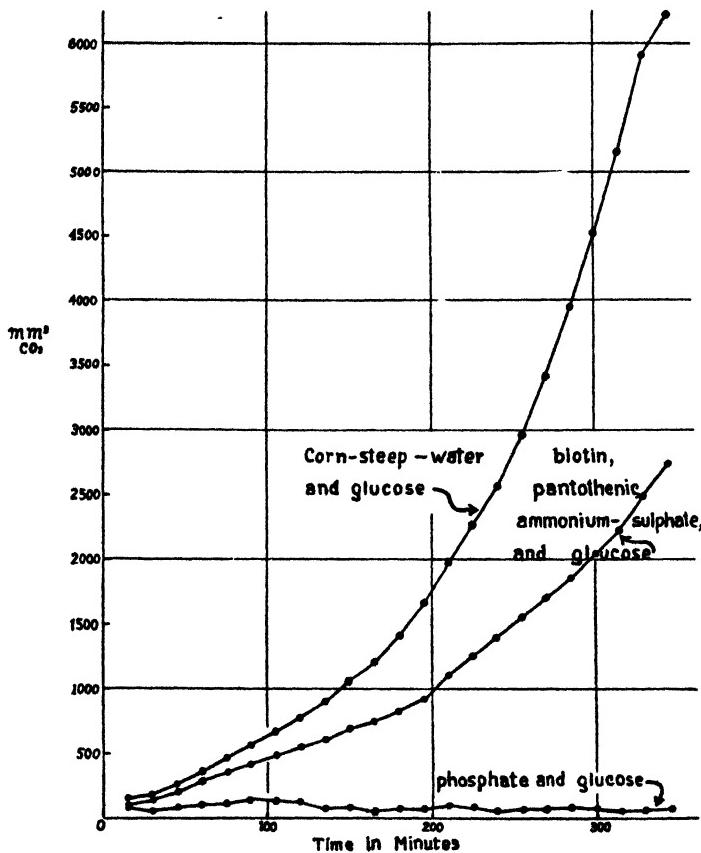


FIG. 13

Curves Showing the Evolution of  $\text{CO}_2$  by Three Comparable Suspensions of Dormant Cells in Different Nutrients over a 345-minute Period

After the granular glycogen had disappeared from the cells grown in 12 or 8% sugar solutions, the cytoplasm still contains the non-granular carbohydrate which is not found in yeasts grown in 1 or 2% sugar.

TABLE I

*The Percentages of Dead Cells and of Cells Containing Glycogen over a 5 Day Period  
in Shallow Broth in Erlenmeyers and in Deep Broth in Test Tubes in  
Nutrients Containing Different Concentrations of Sugar*

Per cent sugar	Day	Erlenmeyer		Final dry wt. <i>mgm. per cc.</i>	Deep tube		Final dry wt. <i>mgm. per cc.</i>
		Dead	Glycogen		Dead	Glycogen	
12	1	1	50	9.43			25
	2	30	100				70
	3	60	100		40	20	
	4	70	40		40	50	
	5	80	20		60	20	3.62
8	1		95	5.62			10
	2		100				50
	3	70	100		25	10	
	4	80	none		15	60	
	5	80	none		40	80	2.99
4	1		90	3.27			50
	2		100				70
	3	20	80		5	95	
	4	50	none		5	40	
	5	70	none		25	40	2.47
2	1		90	2.51			80
	2	0	10				50
	3	0	none		0	20	
	4	0	none		0	50	
	5	0	none		20	20	1.44
1	1		80	1.36			100
	2	0	none				50
	3	0	none		0	5	
	4	0	none		0	1	
	5	0	none		20	5	0.95

*Reduction of Respiratory Activity by Stored Glycogen*

Fifty cc. of nutrient broth containing 1% glucose in 500 cc. Erlenmeyer flasks were inoculated with a heavy suspension of yeast. About 20 hours later, observations showed no budding and no dead cells. Two bakers' yeasts (*S. cerevisiae*, *R* and *U*) and a hybrid (*S. cerevisiae* and *S. globosus*) were used. The contents of some Erlenmeyers were poured into 8 × 1 inch tubes to a depth of about 4 inches (30 cc.) and sufficient 60% glucose solution added to make the total concentration 4% and kept quiet, to produce conditions favorable for glycogen synthesis (samples 1, 2 and 3, Table II). In a second series, sugar

TABLE II

*The Respiratory and Fermentative Activity of Yeasts Containing Visible Deposits of Glycogen and Fat Compared to Normal Cells, in the Presence of 4% Glucose in the Warburg Vessels*

Visible deposit	Sample	Culture	Protein	$Q_{O_2}$	$Q_{CO_2}^O$	$Q_{CO_2}^N$	$Q_{O_2}(N)$	$Q_{CO_2}^O(N)$	$Q_{CO_2}^N(N)$
Glycogen	1	<i>R</i>	33.2	0	60	116	0	1127	2187
Glycogen	2	<i>C</i> × <i>GII</i>	38.7	-52	26	117	-840	419	1885
Glycogen	3	<i>U</i>	33.5	-47	0	83	-880	0	1550
Fat	4	<i>R</i>	56.3	-76	105	322	-850	1162	3580
Fat	5	<i>C</i> × <i>GII</i>	57.5	-127	232	377	-1353	2480	4030
Fat	6	<i>U</i>	50.0	-125	-94	261	-1565	-1170	3261
None	7	<i>R</i>		-105	142	314			
None	8	<i>C</i> × <i>GII</i>		-94	0	150			
None	9	<i>U</i>		-148	0	357			

sufficient to produce a total of 4% was added directly to the Erlenmeyers which were placed on a shaker, to produce conditions favorable for fat synthesis (samples 4, 5 and 6). Tests with Fehling's solution were made and sugar added as necessary in both series. Microscopic examinations were also made to observe the deposition of glycogen and fat. Budding was stimulated by the addition of sugar, indicating that some of the added sugar was used for further growth. After 48 hours, budding had ceased and the 3 yeasts grown in 8 × 1 inch tubes had accumulated glycogen in 100% of the cells. The shaken cultures were all found to contain fat by microscopic observation. The pH of the latter was 8.1 and of the former 4.3.

Samples 7, 8 and 9 were prepared in a heavily aerated broth and the yeast collected before either glycogen or fat had deposited.

The percentage of protein was calculated on the basis of the total nitrogen analysis. This analysis revealed that the yeast containing visible fat deposits contained practically normal amounts of protein, indicating that relatively little fat had been stored during the aeration period. Furthermore, this fat interfered only slightly with the respiratory and fermentative activities as indicated by tests in the Warburg respirometer, since the cultures with no visible deposits had approximately the same activity, at least with regard to  $Q_o$ , and  $Q_{CO}^N$  values. However, the cells containing glycogen deposits had markedly lower  $Q_o$  values. The calculation of the Q values on the basis of the nitrogen content reveals that the cells containing stored glycogen had less than half the activity of those containing stored fat. This fact proves that there has been a real interference with activity rather than a *calculated* effect due to a smaller amount of protein in the cells containing glycogen.

#### SUMMARY AND DISCUSSION

Yeast cells may contain different amounts of carbohydrate and fat reserves. These reserves hinder the respiratory, fermentative and budding activity of the cell. After a long lag, the dormant cells germinate, respire and ferment, and budding of cells is resumed. The low  $Q_o$  values of the dormant cells is quite different from those found in cultures containing large numbers of dead cells. In the early phases of this work, cells were grown in 8% sugar-peptone-yeast extract medium and in this medium, 50 to 90% of the cells died, especially if the cultures were well aerated. The dead cells gave normal  $Q_{CO}^N$  and  $Q_{CO}^O$  values of from 300 to 450, but with  $Q_o$  values of zero. The dead cells were unable to consume  $O_2$ , although they were able to ferment. We produced cultures containing nearly 100% viable dormant cells. These viable dormant cells loaded with reserve materials, were unable to consume  $O_2$  or to evolve  $CO_2$ , but the situation is obviously different from that found when the cultures contain many dead cells.

Winzler, Burk and duVigneaud (1944) have described the "biotin effect." Cultures grown on a biotin-deficient medium are stimulated to respire, ferment and bud by the addition of biotin. They found that the  $Q_o$  values of the cells obtained by growing yeast on a biotin-

deficient medium ranged from zero to very low. The concentration of sugar used in their media is not specified but, even if it were low, one could expect a large percentage of dead cells in a synthetic medium, especially if deficient in biotin. Although synthetic media have a promising future for physiological experimentation, the danger of obtaining diseased cells due to unspecified deficiencies may be considerable in the present state of our knowledge.

The reproducibility of results obtained by the Warburg respirometer depends upon the absence of accumulations of reserve materials in the cells. Cells should be aerated and transferred to the Warburg apparatus when a minimum number of dead cells is present and a considerable proportion is actually budding. In order to make reproducible analyses, growth must be stopped in an active phase and the cells washed with *M/15* phosphate. If the cells are allowed to stand in a nutrient medium, they will accumulate some reserve, either of fat or carbohydrate or both, depending on the aeration and the concentration of sugar.

Accumulated reserves are responsible for the lag in growth observed on the inoculation of the fresh medium. The lag can be completely eliminated if the cells are transferred before any storage has occurred.

Hansen developed a technique of preserving yeast cultures by growing them in 10% sugar broth and allowing the cultures to dry out. The excess of carbohydrate caused growth to cease and storage of reserves brought the cell into dormancy. This procedure could not be used to supply dormant cells in our experiments because a very large percentage of the cells die in high concentrations of sugar and only a few attain full dormancy. Winge (1935) recovered living cells from 50 year old cultures prepared by Hansen.

The fact that dormant cells require a medium containing a relatively full complement of nutrients enables the cells to start growing under conditions that assure continued growth. The fact that they require specific vitamins which they are unable to synthesize may have some importance in solving the problem of inducing other fungal spores to germinate. The conditions for the germination of fungal spores have only been worked out in a relatively small percentage of the species. The use of nutrient media with specific vitamin additions might break dormancy in a number of species whose spores have never been observed to germinate. Mycologists usually study spore germination in hanging drops, often using simply distilled water. Those species

which fail to germinate may yield to the properly selected nutrient solution.

It is a pleasure to acknowledge the skillful and accurate technical assistance of Miss Yulie Kiyasu.

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# The Effect of Electrolytes upon the Thermal Fractions of Alcoholic Acetic Acid Dispersions of Gluten Proteins

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Received July 2, 1945

## INTRODUCTION

Different workers have studied the effects of electrolytes upon certain aspects of the problem of thermal fractionation of gluten protein from alcoholic acetic acid dispersions. Hites (1930)<sup>1</sup> examined the effect of varying the concentrations of different salts on the quantity of protein fractionated at different temperatures from alcoholic acetic acid dispersions of gluten protein.

It was found that  $K_2SO_4$  precipitated substantially more protein than  $CaCl_2$ ,  $KCl$ , or  $CH_3COONa$  at equivalent concentrations. Lowering the acetic acid concentrations increased the quantity of protein precipitated by the salts excepting  $K_2SO_4$ . The influence of temperature on the quantity of protein fractionated from  $N/52$  acetic acid dispersions in 57% alcohol containing  $N/20$   $K_2SO_4$  and  $CaCl_2$  showed that  $K_2SO_4$  caused precipitation at a much higher temperature ( $14^{\circ}C.$ ) than  $CaCl_2$ , which did not show a clear separation of protein until  $-15^{\circ}C.$  had been reached.

Later, Blish and Sandstedt (1935) stated that wheat gluten protein could be fractionated from alcoholic acetic acid dispersions containing a very small quantity of electrolyte by progressively lowering the temperature. Three fractions denoted as glutenin, mesonin and gliadin were separated, and Stockelback and Bailey (1938) concluded that the first two of these were probably individual proteins.

Harris and Johnson (1945) showed that alcoholic acetic acid dispersions of gluten were very sensitive to  $K_2SO_4$  concentration in the temperature region of  $5^{\circ}C.$  Little effect of salt concentration was noted at  $18^{\circ}C$  while, conversely, at relatively low salt concentrations of 30 mgs. per 100 ml. of dispersion, temperature changes had comparatively small effect. Increasing the salt concentration tended to increase protein separation at any temperature level. Little evidence was found to support the hypothesis that distinct protein fractions were removed at any of the temperatures employed.

Gortner, Hoffman, and Sinclair (1929) found a Hofmeister or lyotropic series of ions in increasing peptization, or solubility of wheat flour protein in saline solution.

<sup>1</sup> Thesis presented to the Faculty of the Graduate College of the University of Nebraska in partial fulfillment of the requirements for the M.S. degree.

For anions the arrangement was  $F^- < SO_4^{2-} < Cl^- < C_6H_5O_6^- < Br^- < I^-$ ; for cations the arrangement was  $Na^+ < K^+ < Li^+ < Ba^{2+} < Sr^{2+} < Mg^{2+} < Ca^{2+}$ . The lyotropic effects were measurable at constant hydrogen ion concentration. Docking and Heymann (1939) studied the adsorption of various neutral salts on isolectric gelatin and obtained four lyotropic series. The lyotropic effect of the salts appeared to be composed of an adsorption effect and a salting-out effect.

In view of the results previously secured on thermal fractionation of gluten protein from alcoholic acetic acid dispersions, we decided to study the effect on fractionation of a number of electrolytes at suitable concentrations and to determine whether these would fall into a lyotropic series for either ion. In addition, it was thought desirable to secure confirmatory evidence on a portion of the work of Olcott, Sapirstein, and Blish (1943) dealing with the effect of heat on the physico-chemical properties of dispersed gluten.

### EXPERIMENTAL

Gluten was prepared from a commercial hard red spring wheat flour and dispersed in 0.1 *N* acetic acid with the Waring Blender by the method described by Harris and Johnson (1945). The dispersions employed in the principal investigation were heated at 98°C. for 10 minutes to inactivate the flour proteases (Olcott, Sapirstein, Blish, 1943) and, after cooling to room temperature, were diluted to 50% by weight concentration with ethyl alcohol. The required quantity of electrolyte was then added in *N*/2 solution to 100 ml. of the alcoholic acetic acid dispersions, and the thermal fractionation technique of Harris and Johnson (1945) followed. Four concentrations of electrolytes were used and anions of four different valences were included in the studies. The precipitated protein was removed from the dispersions by centrifuging for 20 minutes in an International Type S.B. centrifuge, followed by decantation of the supernatant liquid from the residual protein. After the removal of the first fractions, it was usually possible to separate the protein precipitating at lower temperatures by filtering the dispersions, and in some instances the protein separated out on the sides and the bottom of the flask and the liquid could easily be decanted without the necessity of centrifuging or filtering. When using the centrifuge with dispersions at a temperature below 4°C. it became necessary to cool the centrifuge bottles to the same temperature level before adding the dispersions to prevent raising the temperature of the liquid with attendant re-dispersion of a portion of the separated protein. This precaution was not required when filtering since the dispersions were not in contact with the funnel sufficiently long for an appreciable rise in temperature to occur. As the more soluble fractions are reached the reversibility of the fractionation process increases.

### RESULTS AND DISCUSSION

The data will be presented chiefly in the form of diagrams rather than tables of precise values because they are not quantitatively

reproducible in different laboratories. Quite definite trends are discernible, however, in the illustrations prepared and little doubt can exist of the implications of the results obtained.

*Effect of Heat on Stabilizing Gluten Dispersions.* Samples of heated and unheated gluten dispersions in 0.1 N acetic acid were allowed to stand 150 hours at 22°C. and 5°C. Samples were then taken for

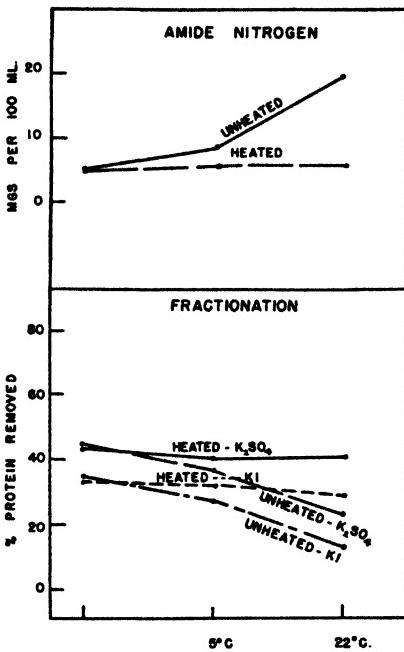


FIG. 1

A Comparison of the Amide Nitrogen Content and Proportion of Gluten Protein Fractionated from Heated and Unheated Dispersions after Storage for 150 Hours at Temperatures of 5°C. and 22°C.

analysis and the results compared with those obtained immediately following dispersion. Very marked increases in non-protein and amide nitrogen associated with a large drop in viscosity were found in the unheated dispersion after standing at room temperature (22°C.) for 150 hours, while standing at 5°C. for the same length of time caused a smaller, though very significant, change in these properties to occur (Figs. 1 and 2). The protein fractionated at 10°C. by  $K_2SO_4$  and KI

solution was also decreased in the two dispersions in the same manner. Little change, however, was evident in the heated dispersions on standing thus fully substantiating the results of Olcott, Sapirstein, and Blish. The fact that less change was observed to take place after 150 hours at 5°C. than at 22°C. is confirmatory evidence that the

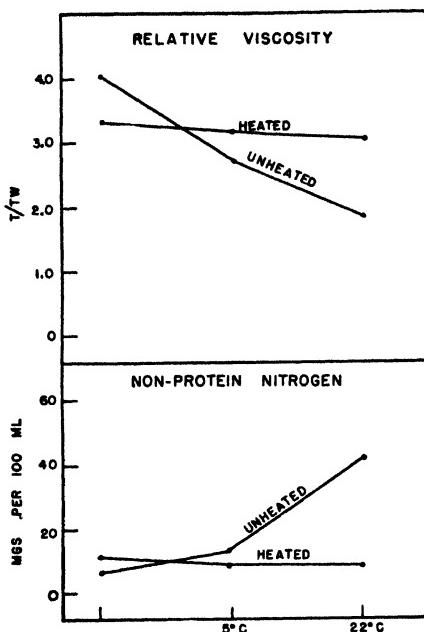


FIG. 2

A Comparison of the Relative Viscosity and Non-protein Nitrogen Content of Heated and Unheated Dispersion after Storage for 150 Hours at Temperatures of 5°C. and 22°C.

changes are induced by an enzyme, since there would be less enzyme activity at the lower temperature. Heating apparently caused a fall in the viscosity of the dispersion; Olcott *et al.* also found heat altered the viscosity of their gluten dispersions.

*Effect of Salt Concentrations.* Fig. 3 shows the influence of salt and salt concentration on the quantity of protein precipitated at different temperatures. Two salts only, sodium chloride and sodium citrate, are shown but the relations between salt concentration, temperature

TABLE I

*Effect of Heat upon Certain Physico-chemical Properties of Gluten Dispersions*

Time of standing	Fractionation results <sup>1</sup>				Amide nitrogen		Non-protein nitrogen		Relative viscosity	
	Heated		Unheated		Heated	Unheated	Heated	Unheated	Heated	Unheated
	K <sub>2</sub> SO <sub>4</sub>	KI	K <sub>2</sub> SO <sub>4</sub>	KI	mgs.	mgs.	mgs.	mgs.	t/tw	t/tw
0 hours	43.2	33.7	44.9	34.7	4.9	5.2	11.2	6.9	3.2	4.1
150 hours 5°C.	40.6	32.1	37.8	27.8	5.7	8.4	9.3	14.3	3.2	2.8
150 hours 22°C.	41.9	29.5	22.9	13.3	5.5	19.4	8.9	42.2	3.1	1.9

<sup>1</sup> Recorded as per cent of total protein.

Note: Amide and non-protein nitrogen shown in mgs. per 100 ml.; viscosity is time of fall of ball in dispersion divided by time of fall in water.

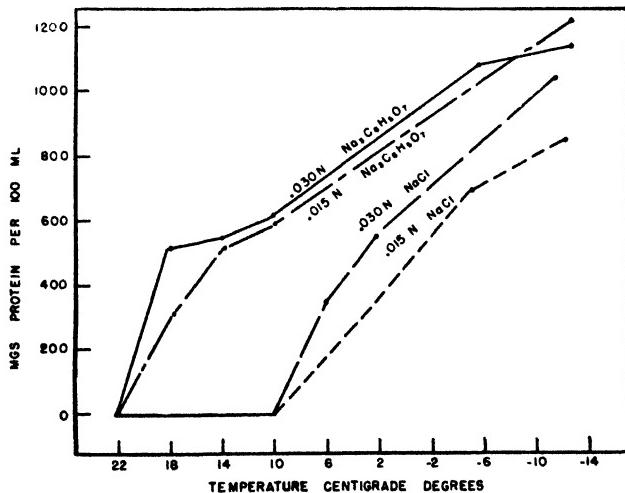


FIG. 3

Effects of Decreasing Temperature and Increasing Electrolyte Concentration upon Total Amount of Protein Removed from Alcoholic-Acetic Acid Dispersion of Gluten Using Representative Mono- and Trivalent Salts

and protein precipitated are representative of the salts studied. The effect of salt used is very marked and shows that differences exist among the salts in respect to their ability to precipitate gluten protein under comparable conditions. This would be anticipated from the work of Hites as well as from a knowledge of the variable effects of these electrolytes in other colloidal systems. In general, however, increasing the salt concentration increases the quantity of protein removed at any temperature, while decreasing the temperature at any salt concentration similarly increases protein precipitation provided there is sufficient electrolyte present to cause precipitation to occur.

*Lyotropic Halogen Series.* At certain temperatures a definite lyotropic series was established for the halogens (Fig. 4). For the four halogen

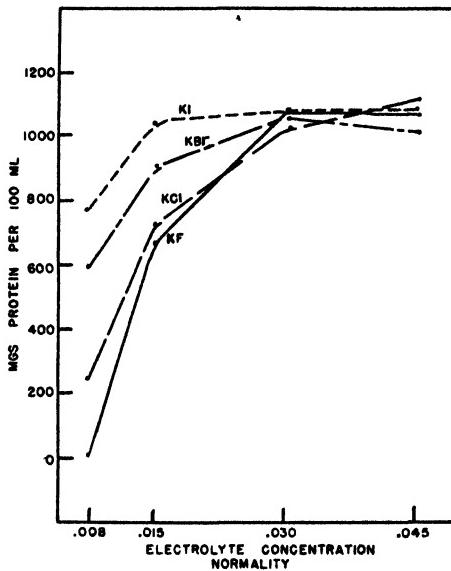


FIG. 4

Lyotropic Effect of Potassium Halides upon Total Amount of Protein Removed from Alcoholic-Acetic Acid Dispersions of Gluten at a temperature of - 10°C.

potassium salts at - 10°C. the precipitating effect was F < Cl < Br < I. Gortner, Hoffman and Sinclair (1929) in their peptization studies of flour proteins found this halogen series to be the most striking

example of the lyotropic effect. At the first salt concentration employed KF had no effect while the influence of the other three salts increased in the order mentioned. At the next salt concentration the effects of the four salts on protein precipitation were more clearly defined, but for the third highest concentration little difference was found among them in the amount of protein removed. It is apparent that the lyotropic effect of the halogens is best brought out at relatively low electrolyte concentrations, even at this low temperature. At more elevated temperatures the precipitating effects of KF and KCl are too weak to be properly evaluated.

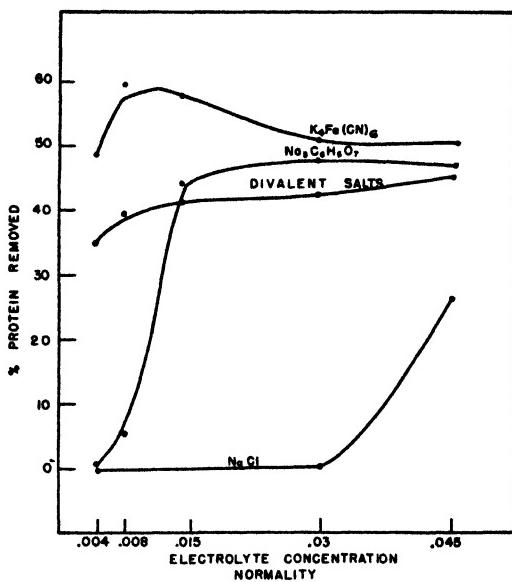


FIG. 5

Comparative Influence of Electrolytes Containing Anions of Different Valencies Upon Total Protein Removed from Alcoholic Acetic Acid Dispersions of Gluten at a Temperature of 10°C.

*Lyotropic Anion Series.* Fig. 5 shows the effect of anion valency of the electrolytes on the total amount of protein fractionated at 10°C. This temperature was used because, at the lower levels employed in demonstrating the effects of the halogen salts, the total quantities of protein removed by  $\text{K}_2\text{SO}_4$ ,  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$  and  $\text{K}_4\text{Fe}(\text{CN})_6$  were large

and quite uniformly high. This tended to mask the lyotropic influence of the anions, as nearly all the protein had been removed from the dispersions before the lower temperatures had been reached. This was particularly true at  $-10^{\circ}\text{C}$ . The slopes of the curves representing the four salts are perhaps as significant as the relative placings of the curves on the figure. The monovalent salt, NaCl, shows the greatest curve rise above  $0.03\text{ N}$  because it has no precipitating effect below a concentration of  $0.03\text{ N}$  at  $10^{\circ}\text{C}$ . The bivalent salts (the curve is a composite of the results secured from  $\text{K}_2\text{SO}_4$ ,  $\text{MgSO}_4$  and  $\text{Na}_2\text{SO}_4$  treatments) show a very slight rise because the effect of the anion at  $0.004\text{ N}$  is so great that further increase in salt concentration has little influence since most of the protein has already been removed. The cause for the very sharp increase in protein precipitated by sodium citrate between  $0.004\text{ N}$  and  $0.015\text{ N}$  is not clear, but may be associated with certain properties of the organic citrate ion. Gortner (1938) stated that variations in ionic radii, degree of hydration, ionic mobility, and the degree of adsorption on the colloid micelle all enter into the total effect of ions of the same valence. The initial hump in the  $\text{K}_4\text{Fe}(\text{CN})_6$  curve is also difficult to explain. The work on these two salts was repeated, with the same results and there is every reason to suspect that this behaviour is characteristic of these salts at this temperature level.

McCalla and Gralen (1940, 1942) have indicated decreasing protein micelle size from the least soluble, or "glutenin" portion, to the most soluble, or "gliadin" component, of gluten. The larger micelles would tend to be precipitated first by diminishing the stability while a larger proportion of the smaller micelles would be progressively removed as the temperature was gradually lowered. An increase in electrolyte concentration or in valency, especially of the anion, would have a similar effect by decreasing the stability conferred by electrical charge. It is probable that these results occur because of changes in the electro-kinetic potential of the protein particles since Briggs (1928) pointed out the importance of this factor in relation to the lyotropic series. There is little doubt that studies with a larger number of electrolytes would demonstrate the lyotropic effect found in other colloidal solutions but, in view of the extensive studies on this problem described in the literature, it did not seem advisable to further extend the scope of the present investigation.

## SUMMARY

A comparison between heated and unheated dispersions after standing for 150 hours at room temperature ( $22^{\circ}\text{C}.$ ) and  $5^{\circ}\text{C}.$  showed marked increase in non-protein and amide nitrogen, and decreases in viscosity and fractionated protein in the unheated dispersion. The effect was most marked in the dispersion kept at room temperature. No significant changes in these properties were found in the heated sample. These results point to a protease adsorbed on the protein particles and inactivated by heating, as found by Olcott, Sapirstein and Blish (1943).

A Hofmeister series of halogen salts was found with regard to effect on protein precipitation at a temperature of  $-10^{\circ}\text{C}.$  This effect was most marked at relatively low salt concentrations ( $0.08\text{ N}$  to  $0.015\text{ N}$ ). In general, increasing the salt concentration increased the quantity of protein removed at any temperature level, while lowering the temperature also increased protein precipitation if sufficient salt were present to initiate protein separation.

Increasing anion valency apparently increased the amount of protein precipitated at any given temperature. This may be explained by a reduction of the positive charge on the protein micelle in alcoholic acetic acid dispersion with a consequent loss in stability.

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# The Dopa Decarboxylase Activity of Human and Animal Tissues<sup>1</sup>

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Received June 29, 1945

## INTRODUCTION

The observation that the amino acid *l*-dopa (3:4 dihydroxyphenylalanine) may be converted to a powerful pressor amine by ischemic kidney tissue (1) has given rise to speculation on the relationship of this phenomenon to hypertension in man. Little information is available on the distribution and activity of *l*-dopa decarboxylase in human tissue and no measurements have been reported on the mammalian placenta. The present investigation was therefore undertaken in connection with studies on the hypertension of human eclampsia.

The enzyme which converts *l*-dopa to hydroxytyramine (3:4 dihydroxyphenylethylamine) was discovered by Holtz, Heise and Lüdtke (2), and its distribution in animal tissues was reported by Holtz in subsequent papers (3, 4). The activity and distribution of this and of other amino acid decarboxylases of mammalian tissues have been reviewed by Blaschko (5, 6), while Oster and Sorkin (7) and Page and Reed (8) have recently considered the relationship between *l*-dopa and arterial hypertension.

To determine the activity of dopa decarboxylase in human tissues it seemed necessary to investigate further the various factors affecting its activity.

## METHODS

Incubations were conducted in modified Thunberg tubes immersed in a water bath at 38°C. and maintained under constant vacuum to the boiling point of water at 38°C. Tissue extracts were prepared by grinding known amounts of fresh tissue in a mortar with quartz sand, adding 10 ml./g. of *M/20* phosphate buffer of pH 7.4. The tissue *brei* was centrifuged at high speed for 20 minutes and an aliquot part of the supernatant fluid was placed in the bottom of the Thunberg tube. Either 5 or 10 mg. of *l*-dopa dissolved in 10 ml. of the same buffer solution were placed in the

<sup>1</sup> Supported by grants from the John and Mary Markle Foundation and the Christine Breon Fund.

side-arm. To the enzyme solution were added 0.1 ml. of octyl alcohol to inhibit amine oxidase activity and 1 ml. of toluol as a bacteriostatic agent. The volume of enzyme solution was then adjusted to 10 ml. by the addition of physiologic saline. After the tubes were evacuated with a pump to remove oxygen, the substrate was tipped in from the side-arm and the incubation continued for one hour. At the end of this time, 4 ml. of 25% trichloroacetic acid were permitted to enter the tube by aspiration so that all enzymatic processes were stopped before allowing oxygen to enter.<sup>2</sup> The protein precipitates were removed by centrifuging. The supernatant fluid was adjusted to pH 4.0 (using Brom-Cresol green as an indicator), filtered and stored in the ice box until ready for bioassay.

For biological assay, cats were anesthetized with intraperitoneal sodium pentobarbital, and a cannula in the carotid artery was attached to a mercury manometer containing a float for continuous kymographic recording. Injections were given in the femoral vein. The extracts were neutralized immediately prior to injection as hydroxytyramine is unstable and is converted to melanin in neutral or alkaline solution. Those amounts of the unknown were injected which produced a series of rises varying from 15 to 80 mm. Hg. Similar amounts of a  $10^{-4}$  dilution of a standard hydroxytyramine hydrochloride preparation<sup>3</sup> were then given, and a log-dose response curve was plotted for both standard and unknown. The relationship between the amine concentration in the standard and unknown was then readily determined (9). By applying the volume corrections, the total yield of amine produced was calculated and expressed in terms of hydroxytyramine hydrochloride. If 100% of the substrate were converted, 5 mg. of *l*-dopa would produce 4.81 mg. of hydroxytyramine hydrochloride, so that the actual percentage of substrate converted in any single incubation is easily calculated. While the sensitivity of cats varies, an average of 30 mm. Hg rise is obtained by the injection of 0.1 mg. of the amine. If the entire unknown sample contained less than this amount, the results were of doubtful significance and were considered negative. The enzyme concentration was then estimated as described below.

## RESULTS

### *Stability of Dopa Decarboxylase*

It would be advantageous to prepare a stable or more purified enzyme, but attempts to do this were only partially successful. Acetone desiccation of guinea pig kidney destroyed the activity completely in four experiments. Drying in the cold by a lyophile process reduced the activity of rabbit kidney extracts by at least 50%, and abolished the activity of guinea pig kidney extracts on two occasions. A stable

<sup>2</sup> Due to imperfect anaerobiosis and despite the addition of octyl alcohol, a small but variable portion of the amine formed (or added in known amounts as a control) was destroyed. A preliminary flushing with oxygen-free nitrogen before evacuation did not remedy this error, so this step was omitted. This loss of amine undoubtedly accounts for some of the observed variations in the calculated yields.

<sup>3</sup> This preparation was kindly supplied by Dr. Gordon Alles.

preparation could be prepared by drying a buffered saline extract of the tissue in a cellophane sac at room temperature and storing the powder *in vacuo*, but the reduction of activity in three such experiments was quite variable. The proteins precipitated between 60 and 80% saturation with ammonium sulfate likewise proved to be active.

Either the intact kidney or its extract could be quickly frozen and would then maintain full activity for two or three days, lose about half of its activity in a week and was usually inactive after two weeks. Toluene was a suitable preservative. In one experiment, Merthiolate in a 1:10,000 concentration appeared to inactivate the enzyme. Dialysis of the extracts for 24 hours at 5 to 7°C. did not reduce the activity and, following dialysis, the activity was not increased by the addition of cocarboxylase (diphosphothiamine) and magnesium ions.

The principal source of human kidney tissue is from necropsies, and since the time elapsing between death and transference of the body to the cold room or between death and autopsy is quite variable, it is important to know the effect of leaving kidneys *in situ* after death on the dopa decarboxylase activity. Observations on the rabbit, the guinea pig and the rhesus monkey indicate that there is a sharp reduction in the dopa decarboxylase activity of renal tissue if the kidneys are left in the body at room temperature for 6 to 12 hours.

One such experiment was designed to determine quantitatively the loss of enzymatic activity under conditions simulating those encountered in the hospital. A guinea pig was killed by a blow on the head and one kidney was immediately removed and extracted. The animal's body remained at room temperature for 6 hours and was then transferred to an ice box at 7°C. for 12 hours before removing and extracting the opposite kidney. Amounts of extracts equivalent to 0.1, 0.25 and 1.0 g. of tissue were incubated with 5.0 mg. of *l*-dopa as described above. Since the curves of amine production indicated that 1.0 g. represented an excess of enzyme, the calculations were based on the lower amounts. In the case of the fresh kidney, 0.1 g. of tissue produced 0.5 mg./hr. of the amine and 0.25 g. of tissue produced 1.8 mg./hr.; whereas equivalent amounts of the kidney which had been left *in situ* after death produced 0.15 mg./hr. and 0.7 mg./hr. respectively, indicating a three-fold loss of activity.

#### *Stability and Activity of Hydroxytyramine*

If a standard 1:10,000 solution of hydroxytyramine hydrochloride containing Merthiolate in 1:10,000 concentration is kept on ice at pH 6.0, it will maintain its potency for many weeks. According to our estimates, it is about one-twentieth as active by weight as epinephrine. The naturally formed amine in the incubated extracts shows the same dose-response characteristics as the standard test solution, and the pressor action of both solutions is potentiated by cocaine and to some extent by the previous injection of *l*-dopa or of

tissue extracts. Repeated rapid injections do not lead to the phenomenon of tachyphylaxis. Both the naturally formed and standard amines are stable to boiling in acid but not with alkali. Holtz *et al.* (2) noted that if the incubation of *l*-dopa with dopa decarboxylase is carried out in the presence of oxygen and vitamin C, the hydroxytyramine is oxidized to dihydroxyphenylacetaldehyde, which is depressor. We confirmed the fact that a depressor product is formed under these conditions. We also noted in 4 experiments that, if octyl alcohol is omitted from the reaction mixture, over half of the amine is destroyed in one hour, indicating that the anaerobic conditions which we were able to obtain could not be relied upon alone to inhibit the amine oxidases. In addition to other tissues, we observed that human placenta destroys hydroxytyramine rapidly, showing the presence of amine oxidase in this tissue.

#### *Variations of Incubation Temperature and of Time*

The action of an excess concentration of dopa decarboxylase upon 10 mg. of dopa for one hour at varying temperatures is illustrated in Fig. 1. No amine production was demonstrated at 0°C., small yields

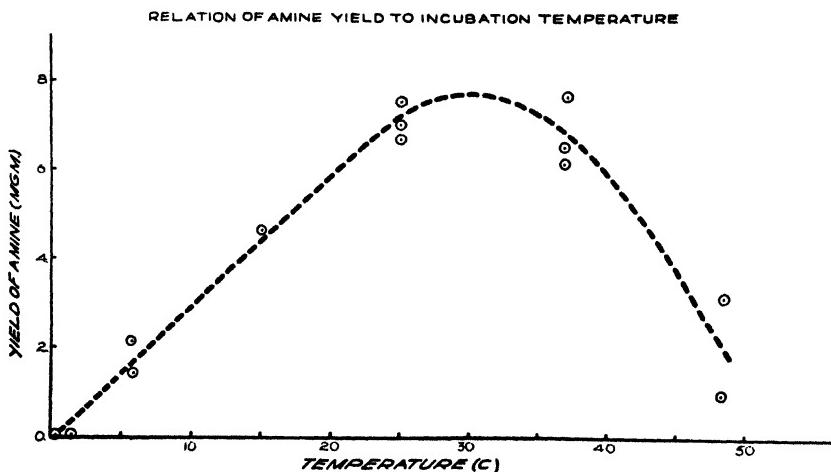


FIG. 1

Relation of Amine Yield to Incubation Temperature

*Time:* 1 hour *in vacuo*; *pH:* 7.2; *Substrate:* 10 mg. *l*-dopa.

*Enzyme:* Extract of 5 g. (excess) of rabbit kidney.

at 6 and 48°C., and maximal yields at both 25 and 37°C. when incubated for 1 hour. A more exact optimum was not determined and all other experiments were arbitrarily performed at 38°C.

Using constant amounts (5 mg.) of substrate, an excess of enzyme and a temperature of 37.5°C., maximal yields of amine were reached between 30 and 60 minutes (3 experiments), and thereafter the yield declined owing to oxidative destruction of the amine (Fig. 2).

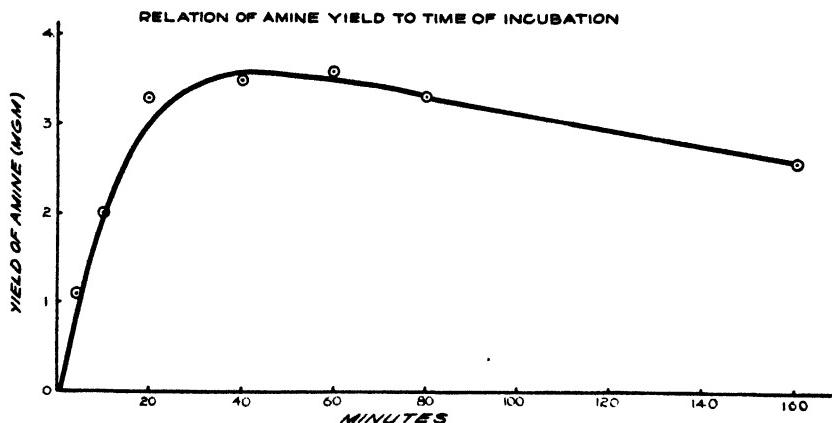


FIG. 2

#### Relation of Amine Yield to Time of Incubation

Temp.: 37.5°C.; pH: 7.2; Substrate: 5 mg. *L*-dopa.

Enzyme: Extract of 1 g. (excess) of guinea pig kidney.

Each point on the curve represents the mean of two experiments.

#### Variations in Substrate and Enzyme Concentrations

In the presence of an excess concentration of dopa decarboxylase, the yield of hydroxytyramine per hour was found to be directly proportional to the amount of substrate within the range studied. The means of 2 such experiments are shown in Fig. 3.

The effects of varying concentrations of dopa decarboxylase upon the conversion of 10 mg. of *L*-dopa to its amine in one hour at 38°C. are illustrated in Fig. 4. The results for three species—guinea pig, rabbit and man—are shown, each curve representing 2 experiments. This was the method used for estimating the relative amounts of dopa decarboxylase in the various tissues. Two or more enzyme concentra-

tions (*i.e.*, two or more quantities of the tissue extracts) were employed for each determination, and if the larger amount proved to be in excess as indicated by a maximal, but less than anticipated yield, then the approximations were made from the results with the smaller

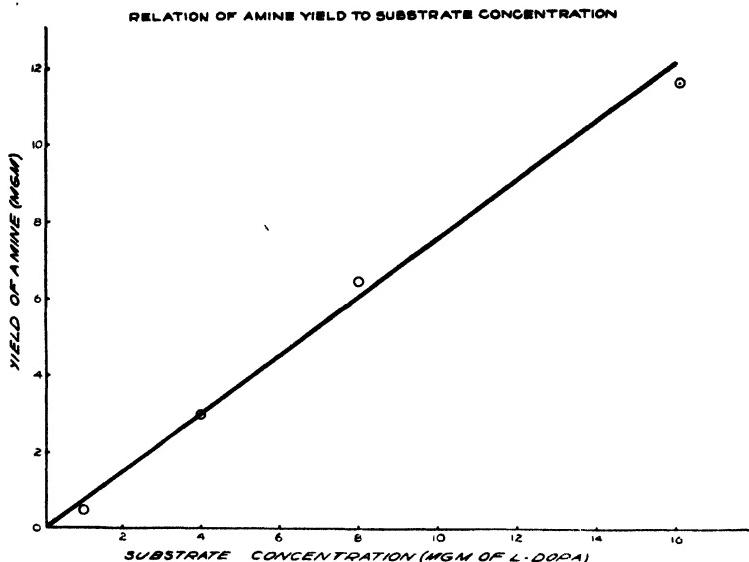


FIG. 3

#### Relation of Amine Yield to Substrate Concentration

*Temp.*: 38°C.; *Time*: 1 hour *in vacuo*; *pH*: 7.3.

*Enzyme*: Extract of 1 g. (excess) of guinea pig kidney.

Each point represents the mean of two determinations.

amount.<sup>4</sup> Enzyme activity is expressed as mg. of amine produced per hour per g. of tissue.

#### *The Activity of Animal and Human Tissues*

The *L*-dopa decarboxylase activity of various tissues as determined by these technics is summarized in Table I and is expressed as the yield of hydroxytyramine produced in one hour by the extract of one

<sup>4</sup> It would be more accurate to utilize an excess of the substrate (100 mg. or more of *L*-dopa) and determine the absolute yield of amine per unit of time, but the unavailability of *L*-dopa at this time prevented us from using this method.

gram of tissue. With small amounts of substrate, the rate of reaction during the first few minutes would obviously be faster than that at the end of an hour, and a truer representation of the rates is expressed by Blaschko (5), who based his calculations upon the carbon dioxide production during the first two minutes. While his values are several

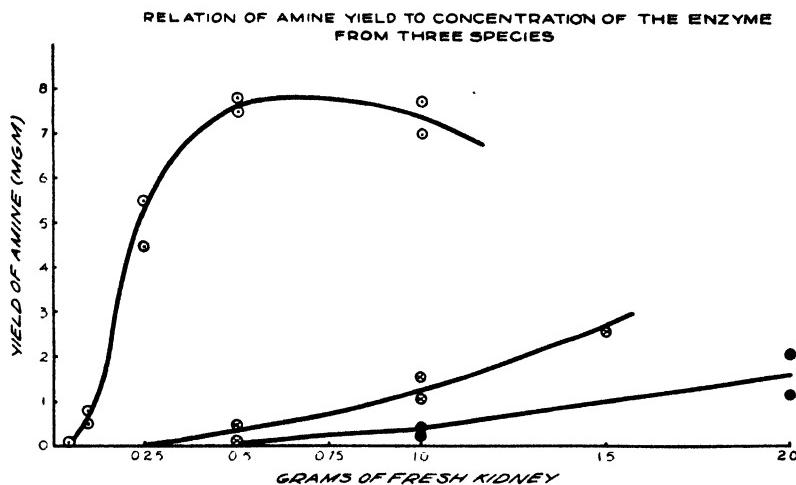


FIG. 4

Relation of Amine Yield to Concentration of the Enzyme from Three Species  
Temp.: 38°C.; Time: 1 hour *in vacuo*; pH: 7.2; Substrate: 10 mg. of *l*-dopa.

The results of two experiments are shown on each curve. This illustrates the method of comparing the dopa decarboxylase content of the various tissues (see text).

○ (Upper curve); Guinea pig kidney. × (Middle curve); Rabbit kidney. ● (Lower curve); Human kidney.

times higher, they show the same variations and similar relationships between the activity of guinea pig, monkey and human kidney tissue.

We found that rabbit and monkey kidneys were about one-tenth as active as guinea pig kidneys but three times as active as human kidneys. The relative inactivity of full term fetal guinea pig kidneys is not unexpected since other enzymes have recently been shown to be very low in fetal tissues (10). Despite the fact that the rat is one of the few animals which responds to the injection of small amounts of

TABLE I  
*The Yield of Hydroxytyramine Produced by Various Tissues*

Species	Tissue	Number	Field (mg./hr./g. tissue)
Guinea pig	Kidney	18	$\pm 11.0$ (Range 2.5 to 24.0)*
	Fetal kidney	2	0.2, 0.0
	Placenta	1	None
Rabbit	Kidney	6	$\pm 1.2$ (Range 0.5 to 1.8)
Monkey	Kidney	2	0.7, 1.0
Rat	Kidney	4	None
	Liver	2	None
	Intestines	1	None
	Brain	1	None
	Lung	2	None
	All viscera	2	None
Human (Autopsy material)	Kidney	9	0.5, 0.12 (none on other 7)
	Liver	1	None
	Intestines	1	None
	Spleen	1	None
(Fresh material)	Kidney	4	0.8, 0.4, 0.2, 0.0
	Placenta (normal)	4	None
	Placenta (eclamptic)	3	None

*Substrate:* 10 mg. *l*-dopa. *Time:* 1 hour, *in vacuo*. *Temp.* 38°C.

Octyl alcohol added. Amounts of amine below 0.1 mg. are considered negative.

\* In the case of the highly active guinea pig kidney, the yields obtained with 0.5 or 0.25 g. of tissue are multiplied by 2 or 4 respectively for the sake of comparison.

*l*-dopa (10–15 mg./kg. intraperitoneally) with a marked rise of blood pressure (8), no *dopa* decarboxylase could be detected in the rat's tissues. In 2 instances, portions of all rat tissues except skin and bones were ground together and tested with negative results.

Only 2 out of 9 human kidneys obtained at autopsy showed any activity. As demonstrated above, the failures may quite fairly be attributed to the length of time elapsing between death and autopsy, usually 8 to 36 hours. In one fatal case of eclampsia, however, the kidneys were removed four hours after death, were kept frozen with dry ice until tested a few hours later, and showed no decarboxylation of *l*-dopa. Among the remaining negative kidneys was one from a woman dying of acute bilateral cortical necrosis of the kidneys.

Of the kidneys obtained at the time of surgical nephrectomy, 3 out of 4 were enzymatically active, the negative one being a hydronephrotic sac with almost complete destruction of the renal cortex.

The placentas from 4 normal pregnancies and from 3 cases of eclampsia were tested in a similar manner, but no dopa decarboxylase activity could be detected. In one of the cases of eclampsia, the placenta was run in duplicate, one portion being homogenized in a Waring blender, dialyzed for 8 hours in the cold, shaken with octyl alcohol before testing, then incubated anaerobically with 25 mg. of *l*-dopa, but no measurable amount of amine was produced. From the negative results on the eclamptic kidney and placental tissue, it would seem unlikely that dopa decarboxylase activity could be responsible for the hypertension which is such an outstanding feature of that disease.

This work was made possible by the technical assistance of Miss Dean Mayell, Mrs. Racheal Reed and Mr. George Feigen, and the helpful advice and criticism of Drs. Gordon Alles, David Greenberg and John Eiler.

#### SUMMARY

The amino acid, *l*-dopa, is converted anaerobically by dopa decarboxylase to hydroxytyramine, and it is believed by some that this amine may play a role in the production of renal hypertension. The methods for the biological estimation of dopa decarboxylase in tissues are described, together with some observations on the stability and the properties of this enzyme.

Guinea pigs contain a high concentration of the enzyme in the adult kidney, but not in the full term fetal kidney nor in the placenta. The kidneys of rabbits and monkeys are about one-tenth as active as those of the guinea pig. No dopa decarboxylase could be detected in any tissues of the rat.

Human kidneys contain a lower concentration of dopa decarboxylase than those of the rabbit or monkey, but no activity could be found in the human placenta.

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## Book Reviews

**A Symposium on Mammary Tumors in Mice.** By Members of the Staff of the National Cancer Institute. Edited by FOREST RAY MOULTON. American Association for the Advancement of Science. Washington, D. C., 1945. 223 pp. Price \$4.-

Of the various tumors observed in animals, mammary cancer of the mouse became usable for experimentation after the possibility of its hereditary transmission was established. By inbreeding, M. Slye isolated strains of mice the females of which frequently develop mammary cancer. Similar hereditary cancerous strains were also obtained in other laboratories and the opportunity was thus originated for studying this peculiar pathological condition of the mouse.

This Symposium gives a compilation of the collected data concerning spontaneous mammary tumors of the mouse. A. J. Dalton, T. B. Dunn, G. H. Algire and H. W. Chalkley describe their anatomy, and W. E. Heston summarizes their genetics. M. B. Shimkin discusses "Hormones and Mammary Cancer in Mice," H. B. Andervont the "Milk Influence" in their genesis, and H. P. Morris brings "Diet and Some Other Environmental Influences in the Genesis and Growth of Mammary Tumors in Mice." The contribution of J. P. Greenstein includes "The Chemistry of the Mammary Tumors," and that of H. M. Dyer "Experimental Treatment of Mammary Tumors in Mice." M. B. Shimkin has written the introductory and concluding remarks.

The book will be very useful for any investigator seeking a rapid orientation in the actual status of the problems discussed.

The spontaneous mouse cancer is especially suitable for experiments on the complex problem of its etiology. Four groups of factors are involved in its genesis: the hereditary ("genetic") factor, the hormonal factor (the tumors are observed in females only), the milk factor (they can be transmitted through the milk of the lactating female), and the unknown additional factor which brings about development of the tumor in the animals predisposed by the previously enumerated factors.

The "milk factor," discovered by J. J. Bittner in C. C. Little's Jackson Memorial Laboratory, can be transmitted by cell-free material. It is thermo-unstable, appears to be of a protein nature, and its features suggest the character of a virus. The article of Andervont, who himself contributed much in this field of research, is especially valuable for orientation in that chemical problem.

In other respects the collected facts concerning other chemical problems bring "little if anything which sets these tumors apart in any way from the chemistry of all other kinds of tumors."

Male mice of the cancerous strain when injected with estrogens also develop spontaneous breast cancer (Lacassagne). From this fact conclusions are often drawn that estrogens may provoke human cancer. This is unfounded, for the essential circumstance leading to the appearance of tumor is, in this case, the "change in sex"

of the animal brought about by the excessive dose of estrogen applied. Analogous "changes of sex" occur very rarely, if at all, in man.

It should be remembered that analogies drawn from findings on spontaneous tumors of the mouse can only be applied very cautiously to human cancer, in contrast to the results obtained from studies on experimental mouse cancer, which furnish important additions to our knowledge of cancer.

This circumstance, however, does not discourage the authors who believe correctly that concentration upon one particular type of cancer in one particular animal species cannot fail to contribute toward further research efforts in the biological problems concerned.

HENRY K. WACHTEL, New York

**Microbial Antagonisms and Antibiotic Substances.** By SELMAN A. WAKSMAN, Professor of Microbiology, Rutgers University; Microbiologist, New Jersey Agricultural Experiment Station. The Commonwealth Fund, New York, N. Y., 1945. ix + 350 pp. Price \$3.75.

This book, which summarizes what is known about microbial antagonisms and antibiotic substances, is important and timely. It is the first book of its kind to attempt an ordered presentation of the wealth of information available in this field.

In the early chapters the author presents at length the observations which have been made on soil organisms in general, starting with their natural habitat and the influence of various natural factors upon their environment. The important phenomenon of the survival of pathogenic organisms in the soil is discussed and the complex field of interrelationships exhibited among a mixed soil population with their various competitive, associative and antagonistic relationships. In those chapters which survey and emphasize the different phenomena of antagonism among the soil organisms the literature dating back to Louis Pasteur is treated exhaustively, a fact which will be greatly appreciated by the specialist. From this the author proceeds to the description of the different procedures used in the isolation and cultivation of the various antagonistic microorganisms and the different methods devised for measuring the activity of the antibiotic substances. Proper emphasis is laid upon the fact that a number of organisms are capable of producing more than one antibiotic substance and upon the usefulness of the antibiotic spectrum in distinguishing between the different antibiotics. The following sections discuss bacteria, actinomycetes and fungi, respectively, as antagonists, the chapters forming a general synopsis of all the work carried out in this field from the earliest times to the present.

A wealth of material is presented and supplemented with many tables and figures.

Although emphasis is laid upon the soil organisms as antagonists to human and animal pathogens, due consideration is given to the antagonistic relationships of the soil organisms among themselves. Completion of the subject is accomplished in the following chapters describing the microscopic animal forms as antagonists and finally the as yet unknown field of antagonistic relationships among soil organisms and viruses is touched. Consequently, all that has been published relating to the chemical and biological properties of the known antibiotics is summarized. Detailed information is given concerning the cultural procedures for the production and methods

used for the purification and isolation of the active substances. In discussing the nature of their action upon the morphology and physiology of bacteria, whether bacteriostatic, bactericidal or lytic in character and comparing these observations with the mechanism of action of disinfectants the modern conception of combating disease is shown.

The chapter describing the utilization of the antibiotics for the control of disease presents the experimental and clinical data which have been compiled with respect to the toxicity and therapeutic applications of the various antibiotic substances of soil origin. Of these, penicillin, which has been the most effective and exploited antibiotic to date, has yielded the most information and is consequently the most discussed. But the opportunities and likelihood that other antibiotics already discovered will be effective where penicillin is not are optimistically reviewed. The following chapter dealing with the microbiological control of soil-borne plant diseases, especially fungi, offers another useful and important application of the antibiotics and antagonistic organisms. The final chapter shows the beginning of the modern development in the field of antibiotics and outlines the tasks which lie ahead for the microbiologists, chemists, physiologists and clinicians who are or may become engaged in the study of new antibiotics and presents an optimistic view of the benefits which their combined work will bring to mankind. An appropriate classification of the antibiotic substances, a glossary and a valuable bibliography containing over 1000 references conclude the book.

The author's point of view is that of a soil microbiologist and this view predominates throughout the book and lends it its typical imprint which by and large is to the advantage of the book. Considering the wealth of information which had to be gathered and organized, the author has done a great job well. The structural organization of the book is well chosen and the different chapters form a harmonious and logical sequence. The many tables and figures included enhance and add greatly to the insight.

Notwithstanding the outstanding contribution this book makes, the reviewer feels bound to express a few critical remarks. Some of the tables (pp. 70, 77, 141, 192) where the efficacy of antibiotic agents is elucidated are not clear because pure substances are compared with partially purified crude culture extracts. The author mentions in other places (p. 199) that "confusion often resulted from the use of crude preparations." A note on the degree of purification of the substances in question would have been in order. Objection should also be raised to the classification of antibiotics, which have not yet been isolated, as soluble in water, alcohol, ether, etc. (pp. 160, 171), because such a classification is both premature and confusing. Apart from the fact that such a classification is based chiefly on the properties of the antibiotic in crude culture, where the actual properties of the substance may be masked (*e.g.*, presence of two different antibiotic substances, salt formation, etc.) the only classification that will be a correct one is the chemical classification. The biochemist would have benefited by some data on the degree of purity in which the antibiotics are found in crude culture filtrates as contrasted with the potency of the isolated substances. The author furthermore uses the term "isolation" in instances where the correct designation would be: purification, concentration, etc. (pp. 114, 117, 120, 174).

The last objection, and possibly the strongest one, is that the book is too much on the *relata referro* side. There are too many quotations from original articles, some without quotation marks. A more critical exposé of the many mentioned facts, especially of the older literature, would have made the book more valuable. It would be a mistake to overemphasize these and other minor limitations. The wealth of material presented and summarized makes the book a valuable one. Considering the present rate at which the antibiotic field is developing and changing the author will have to rewrite the book soon if it is to be kept up to date.

WALTER KOCHOLATY, Philadelphia, Pa.

**Soil and Plant Analysis.** By C. S. PIPER. Interscience Publishers, Inc., New York, 1944. xiv + 368 pp. Price \$4.50.

During the latter half of the nineteenth century, agricultural chemists carried out enormous numbers of analyses of soil and of the crops grown thereon, in the belief that plant analysis would indicate crop needs for the various elements and that soil analysis would provide information as to the supply of those necessary elements. It then became apparent that fertility is not expressible in terms of a simple balance sheet, and for a time the purely analytical approach became discredited. The chief difficulty was found to lie in the fact that the availability of the plant nutrients in the soil cannot well be determined by ordinary analytical means, and that, in any case, it is the rate of nutrient release and not the actual concentration at any one time that is of dominant importance. Of more recent years, however, it has become apparent that certain chemical and physical determinations may be used to characterize soils and that, having such information, certain deductions can be made as to the behavior of that soil. For example, a knowledge of the silica/sesquioxide ratio of the clay fraction, by indicating whether predominantly kaolinitic or montmorillonitic clay minerals are present, also permits conclusions as to probable physical characteristics and chemical reactions, such as anion fixation. Similarly, the determination of percentage base saturation of a soil allows an accurate estimate of its lime requirement.

This book is a compilation of methods of soil and plant analysis used extensively in Australia, chiefly at the Waite Agricultural Research Institute. In the words of the author, "The experience gained in handling numerous soil samples, which have been collected from all parts of Australia . . . has been particularly valuable, since it has necessitated the adoption of methods which will give dependable values when applied to these widely varying soil types. Methods originally developed for a single soil type have often been found to be inapplicable, without suitable modification, to the wide range of soils encountered in Australia." It is not surprising that many of the new developments in the rapidly enlarging science of pedology have arisen amongst groups of workers who normally have access to and contact with the great variety of zonal and intrazonal soils found in continental areas, such as Russia, the U. S., and Australia, where well-developed soil characteristics reflect the influence of a great range of climates and accompanying vegetative patterns. Analytical procedures subjected to such a test are likely to be found highly reliable.

Methods of analysis and the various steps in the operations involved are given with commendable detail. In many cases, the necessary basis for calculation also is

provided. The selection of procedures appears to be rather more conservative in the case of soil analyses than plant analyses. For example, in most laboratories, the glass electrode has long supplanted the quinhydrone electrode for the determination of soil reaction, and few of the "single value physical constants" are in general use. Full use is made of the newer organic reagents in the determination of such soil and plant constituents as iron and titanium (cupferron), magnesium (hydroxyquinoline), cobalt, copper, zinc (dithizone), or boron (quinalizarin). There is little question that this volume will be most helpful to all analysts concerned with soils or inorganic plant constituents.

A. G. NORMAN, Ames, Iowa.

**Physiology and Biochemistry of Grain in Storage.** By V. L. KRETOVICH, Professor of Biological Chemistry in the Biochemical Institute of the Akad. Nauk S.S.R. Published by Akad. Nauk S.S.R., Moscow, Leningrad, 1945. 136 pp. Price 7 rouble.

The need for a systematic and critical Russian review of the physiological and biochemical changes occurring during ripening and storage of grain arose with the expansion of agricultural facilities in the U.S.S.R. The present survey of data published in the Russian and non-Russian literature is intended to fill this gap.

Deposition of dry substance in grain, changes in the chemical composition of grain during ripening, the rôle played by the fructosans in the accumulation of starch, and the changes occurring in nitrogenous substance and in enzymes during ripening are discussed in the chapter dealing with the biochemistry of grain ripening. Another chapter is devoted to the damage of grain by frost and the wheat bug. The physiological condition of grain in storage, the activity of the enzymes encountered in grain and the energy of the occurring biochemical processes depend on the combined action of humidity, temperature and aeration (chapters 4 and 5). The after-ripening of grain is favorably influenced by decreasing the relative humidity of the ventilating air and by drying at moderate temperatures. This increase the germinating capacity, decreases the activities of catalase and amylase, and improves the bread-baking properties. Grain storage over long periods of time or under unfavorable conditions is accompanied by a weakening of the respiratory metabolism of the grain as well as by a change in the activity of the oxidation-reduction enzymes of the grain, particularly the dehydrogenases.

Of the rather extensive bibliography of 11 pages, the first 4 pages are devoted to Russian authors. A few of the Russian papers were published in German periodicals.

G. BEREND, Nutley, N. J.

**Microbiology of Foods.** By FRED W. TANNER, Professor of Bacteriology in the University of Illinois. Garrard Press, Champaign, Illinois, 1944. viii + 1196 pp. Price \$12.50.

The second edition of Tanner's *Microbiology of Foods* represents a considerable increase, both in size and in scope of material, over the first edition which appeared 10 years ago. This edition is truly a comprehensive compilation of the literature dealing with the microbiology of food manufacture and preservation. The text runs to 1200 pages, divided into 26 chapters, some of which are documented by as many as 500 references with an over all total of perhaps 5000. The author's aim has been

to supply a key to the literature in this field. The methods in use, which have been published in various manuals dealing with the bacteriological examination of water, milk, etc., have been reprinted in the book.

For purposes of review, the text may be divided roughly into four sections. The first quarter of which deals with the principles of bacteriology involved in food preservation, the main methods of study of preservation and spoilage, and detailed discussions of the bacteria, yeasts and molds which are encountered in foods. The more general phases are followed by a very complete chapter (about 100 pages) on the bacteriology of water and sewage.

The second quarter of the book (seven chapters) is devoted to the bacteriology of milk and milk products, including sanitation, manufacture, and preservation. This section is considerably expanded from the first edition and is well documented. A number of errors are noted, such as the interpretation placed on the names of organisms from the older literature. The errors noted are, however, surprisingly few for a text of this magnitude.

Approximately the third quarter of the book deals with the microbiology of foods other than milk. This includes fruits and vegetables, fruit and vegetable products, fermented foods, and meat, eggs, and fish. Entire chapters are devoted to those foods which constitute special cases. These include tomato products, bread, and sugar and sugar products. A chapter on intestinal bacteriology contains the material not discussed under water and sewage.

The last quarter of the text contains a chapter of 150 pages dealing with canned foods, their preparation, processing, and causes and detection of spoilage. Both commercial and home canned foods are included. A full chapter of about 100 pages is devoted to the microbiological methods for vitamin assay. This chapter contains an excellent collection of the available methods and their application to foods. The more pertinent data regarding the occurrence and properties, and where known the function, of the vitamins are tabulated. The last chapter is a description of the media used in the examination of foods. Considerable detail is given to the preparation and to the particular tests in which most of the media are used.

The bulk of the book precludes its use in the average food bacteriology or applied bacteriology course as a text. It does, however, serve as a reference and source book for advanced students and workers in the food preservation industries.

I. C. GUNSAULUS, Ithaca, N. Y.

**Photosynthesis and Related Processes, vol. I.** By EUGENE I. RABINOWITCH, Research Associate, Solar Energy Conversion Research Project, Massachusetts Institute of Technology. Interscience Publishers, Inc. New York, N. Y. 1945. xiv + 599 pp. Price \$8.50.

This long anticipated treatise on photosynthesis is a thoroughly worthy successor to the A.C.S. Monograph by Spoehr which was the outstanding reference and research workers' textbook from 1926 until the publication of the present comprehensive and critical volume of Rabinowitch. The striking quality of Rabinowitch's book is its clarity and its complete coverage of the pertinent data in related fields of physics, thermodynamics, photochemistry and certain aspects of biology. In the beginning the scale and significance of the subject is discussed and is followed by an admirably

lucid account of the early history of the discovery of photosynthesis. Part One then deals with the chemistry of photosynthesis and related processes. Here the over all reaction is described and the various products are considered in detail. The related processes of oxygen production by leaf powders and isolated chloroplasts, the photochemical oxidation of water, the chemical reduction of CO<sub>2</sub>, photo-, and chemosynthesis of bacteria, and the H<sub>2</sub> metabolism of certain algae are described. The various theories of the mechanism of photosynthesis proposed in the past are then clearly presented and classified with a uniform terminology and illustrative diagrams. This treatment should dispose of most of them for all time. Carbonate-bicarbonate equilibrium and CO<sub>2</sub> fixation by plants is then taken up and is followed by a consideration of possible intermediate photosynthetic products. Particularly in this section as well as throughout the book excellent use is made of thermodynamic data for deciding as to the relative plausibility of various hypothetical reaction pathways. For the thermodynamic calculations for various biochemical substances the book is very valuable and will therefore be useful to those interested in other metabolic processes. The respiratory mechanism of plants which many biochemists would consider to be intimately related to photosynthesis are given but a few pages mainly in the chapter on intermediates. There is little consideration of the properties of the known plant enzymes which catalyze related biochemical reactions. As this treatise will be used as a handbook for research workers it might profitably have included some experimental details and descriptions of procedures. This omission does, however, allow space for greater clarity in the exposition of theoretical matters. The discussion of O<sub>2</sub> liberation and the possible participation of peroxides is followed by two chapters on the inhibition and stimulation of photosynthesis by added chemical agents.

In Part Two the structure and chemistry of the constituents of chloroplasts are skillfully handled. These chapters far surpass any text on plant physiology for their clear, detailed and critical presentation of chloroplast composition, structure, and pigment chemistry. The chemistry of plant pigments is included in sufficient detail to give a good description of the structure and reactions of the pigments and their derivatives. The organic chemists' method for proving the structures have wisely been omitted as irrelevant to the main purpose of the book. Rabinowitch's own work on the reversible chemical and photochemical bleaching of chlorophyll and the photochemistry of chlorophyll sensitized reactions *in vitro* and *in vivo* is well handled. A final chapter on the relation between photosynthesis and respiration and the effects of light on plant respiration brings Volume One to a close. Volume Two which is expected to appear within a year is planned to cover the spectroscopy and fluorescence of the pigments and the kinetics of photosynthesis. Both the author and the publishers are to be greatly commended on the successful completion of such a large undertaking as the publication of this work. The physicist and the physical chemist will find much to stimulate thought on the application of their methods to a fundamentally important chain of reactions that has so far not been adequately investigated. The biochemist will be disappointed at the lack of specific knowledge of the photosynthesis enzymes and intermediates whose only evidence for existence is based on the physico-chemical interpretation of kinetic measurements. It is to be hoped that this book may stimulate a few biochemists to enter this field which has so long been neglected.

by them. Fourteen proof reader's errors were encountered none of which are seriously misleading. This volume can not long remain unread, at least in part, by everyone interested in the physico-chemical interpretation of physiological processes. The relevant chapters are to be highly recommended as textbook material for mature graduate students in general physiology, plant physiology, biochemistry, bacteriology, and photochemistry.

C. S. FRENCH, Minneapolis, Minn.

# The Heat Labile Growth Factor for Paramecium in Pressed Yeast Juice<sup>1</sup>

W. H. Johnson and E. L. Tatum<sup>2</sup>

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Received August 1, 1945

It has long been recognized that many of the more specialized protozoa, normally feeding on other living organisms, require a heat labile substance or substances for growth. The use of media containing heat-killed bacteria and other heat-treated ingredients alone will not support growth of such organisms. The nature of this problem has been discussed in reviews by Rees, Reardon and Jacobs (1941) and by Johnson (1941).

Recently, two such ciliates have been grown in the absence of other living organisms. *Colpoda duodenaria* has been cultivated successfully in a bacterial plasmolytate preparation (Taylor and van Wagendonk, 1941; van Wagendonk and Taylor, 1942; Tatum, Garnjobst and Taylor, 1942; Garnjobst, Tatum and Taylor, 1943). *Paramecium multimicronucleata* has also been grown in media containing unheated pressed yeast juice (Johnson and Baker, 1942). This paper is a further report on the growth of *Paramecium* with fractions of the yeast juice.

## EXPERIMENTAL

**Methods**—The pressed yeast juice (p.y.j.) was prepared according to the method described by Johnson and Baker (1942). However, instead of using all the juice pressed from Fleischmann's bakers' yeast, the first half of the juice (about 60 cc. per pound) was discarded. The remainder of the expressed juice has greater activity and is more easily filtered.

For sterilization of the juice, three types of bacteriological filters were tried: Corning glass filters, Seitz filters and Chamberland candles. Sterile active preparations have been obtained with all of these, but filtration through a glass filter was very difficult and the filtrate was not as active as the juices from the other two types of filter. The Chamberland candles soon became clogged, and the Seitz filter with serum No. 1 pads was thus found to be the most practical. The juices filtered through

<sup>1</sup> Work supported in part by grants from the Rockefeller Foundation.

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the glass filters and through the candles were always sterile, whereas the Seitz-filtered juice occasionally had to be refiltered. Each filtration through a Seitz filter reduced the activity of the juice. Juice filtered through three No. 1 pads had only about half the activity of juice filtered through one pad.

Fair growth of *Paramecium* was obtained when the p.y.j. was added to triple distilled water or to Osterhout's medium, but better growth was obtained when the yeast juice was added to 0.5% proteose-peptone. The medium selected for maintaining the cultures contained 1 part of p.y.j. to 20 parts of 0.5% proteose-peptone. All cultures were grown in an incubator at  $25^{\circ} \pm 1^{\circ}\text{C}$ .

Sterility tests, as described by Johnson and Baker (1942), were made regularly on all juices and fractions used, and on each culture.

*Properties of Pressed Yeast Juice*—The p.y.j. is relatively stable. When stored in a refrigerator at  $10^{\circ}\text{C}$ . for four or five months it still permitted good growth of *Paramecium*. When this old juice was used, the cultures grew more slowly at first but they ultimately attained the same maxima in numbers. Buchner, Buchner and Hahn (1903) first reported the proteolytic activity of p.y.j. They found that the juice would liquefy coagulated p.y.j. and other coagulated proteins and we have made the same observations. They also noted that crystals of tyrosine were formed in juice which had been kept for some time. In most of our tubes of sterile juice, crystals of tyrosine appeared after ten days to two weeks. However, the growth-promoting properties of the juice were not impaired by this proteolysis. Fraction 3 was a clear, slightly straw-colored liquid and no tyrosine crystals appeared after standing in the refrigerator for six weeks. Furthermore, when a sample of fraction 3 was added to some of the coagulated p.y.j. there was no liquefaction, indicating that the proteolytic activity of the juice had been destroyed or lost in the precipitation.

The yeast juice is heat-labile. Tests were made in which samples of the diluted juice (1 : 20) were heated for 30 minutes at  $40^{\circ}$ ,  $50^{\circ}$ ,  $55^{\circ}$ ,  $60^{\circ}$ ,  $65^{\circ}$ , and  $70^{\circ}\text{C}$ . Juice heated at  $55^{\circ}\text{C}$ . and above was inactivated. The loss of activity was roughly proportional to the degree of protein coagulation. Juice which had been inactivated by heating at  $55^{\circ}\text{C}$ ., as judged by immediate tests, supported fair growth after storage at  $10^{\circ}\text{C}$ . for two weeks.

*Dialysis of Pressed Yeast Juice*—When the sterile juice was dialyzed for 18 hours against equal volumes of sterile distilled water and the two fractions were tested for growth, the dialyzates had no significant activity while the undialyzable fractions gave very good growth. The results of one such test are given in Table I. In another test, the sterile p.y.j. was exhaustively dialyzed against running distilled water. In this case, the undialyzable fraction alone gave no growth when added to the proteose-peptone medium, but very good growth was obtained in this medium when supplemented with a small amount of autoclaved p.y.j. from which the coagulated proteins had been removed. This medium contained 5 cc. of 0.5% proteose-peptone, 0.5 cc. of the un-

TABLE I  
*Growth of Paramecium in Fractions of P.Y.J.*

Fraction Tested		Dry weight of fraction per culture in mg.	Initial number of paramecia	Final number of paramecia	Time in days
Number	Total dry weight in g. per 50 cc. p.y.j.				
Control	—	—	21	500	10
1	—	—	15	60	10
2	—	—	9	1625	10
1 and 2	—	—	18	1625	10
Control	4.56	22.5	12	900	14
3	0.73	5.0	15	1250	14
4	2.98	30.0	18	440	14
5	0.11	2.5	50	285	14

Controls contained untreated p.y.j.

Fraction 1: dialyzate of p.y.j.

Fraction 2: undialyzable fraction of p.y.j.

Fraction 3: fraction precipitated three times with  $\frac{1}{2}$  saturated  $(\text{NH}_4)_2\text{SO}_4$ .

Fraction 4: unprecipitated fraction from the first precipitation of fraction 3.

Fraction 5: fraction precipitated from 4 by saturated  $(\text{NH}_4)_2\text{SO}_4$ .

All fractions containing  $(\text{NH}_4)_2\text{SO}_4$  were dialyzed against running distilled water and sterilized by filtration before testing.

dialyzable fraction of p.y.j., and 0.25 cc. of autoclaved p.y.j. The autoclaved p.y.j. apparently contains a heat-stable substance, or substances, necessary for the growth of *Paramecium*. It is not present in the thoroughly dialyzed p.y.j. nor in the proteose-peptone. This autoclaved yeast fraction alone or with proteose-peptone, will not support growth of *Paramecium*.

*Precipitation of P.Y.J. with Ammonium Sulfate*—The preceding tests indicated the possible protein nature of the essential heat-labile substance in p.y.j. In a preliminary experiment to further test this possibility, it was found that the active material was precipitated from p.y.j. at three-fourths saturation with ammonium sulfate. The precipitated fraction, when dissolved in distilled water, dialyzed and filter-sterilized gave good growth when added to the proteose-peptone medium containing a small amount of the autoclaved fraction of p.y.j. Further tests showed that the material which had been purified by

three repeated precipitations with ammonium sulfate gave very good growth. This is shown in Table I.

The growth obtained with fraction 3 was better than that obtained in the control. Tests were made in which fraction 4 was added to fraction 3, and in which fraction 5 was added to fraction 3, with no supplementary effects. Fraction 3 undoubtedly contained most of the active material. This fraction coagulated readily upon heating and was not active after autoclaving.

*Requirement of Heat-Stable Yeast Fraction*—Neither fraction 3 nor the exhaustively dialyzed juice would support growth of *Paramecium* unless a small amount of the autoclaved fraction of p.y.j. was added. Some preliminary tests have been made to see whether this fraction of autoclaved juice could be replaced by known vitamins. A mixture<sup>3</sup> of vitamins B<sub>1</sub>, B<sub>2</sub>, B<sub>6</sub>, *p*-aminobenzoic acid, inositol, calcium pantothenate, nicotinic acid, choline and biotin was tried with fraction 3. After three weeks, the organisms in these cultures appeared healthy and had divided once or twice, but no significant growth had occurred. Further experiments are necessary to determine whether these vitamins with other known substances can replace the essential heat-stable factor or factors in the autoclaved fraction of the p.y.j. When the mixture of vitamins was added to control cultures with untreated p.y.j., no increases in fission rates nor in the maximum populations were noted, but the paramecia in such cultures remained alive and active for two to three weeks after the organisms in similar cultures, but without the added vitamins, had all died. This seems to indicate that one or more of these vitamins plays a role in the nutrition of *Paramecium*.

*Relation of the Heat-Labile Growth-Factors in P.Y.J. and in Plasmoptryzate*—The two ciliates, *Paramecium* and *Colpoda*, require heat-labile substances for growth. van Wagendonk and Taylor (1942) reported that *Colpoda duodenaria* grew in either pressed yeast juice or in bacterial plasmoptryzate medium, but did not grow in Lebedew juice. The activity of p.y.j. for *Paramecium* and the apparent inactivity of Lebedew juice for this ciliate in our experiments, suggested that the heat-labile factors in p.y.j. and in plasmoptryzate might be similar. This possibility was therefore investigated.

<sup>3</sup> The final medium contained in γ per cc.: B<sub>1</sub> and choline, 1; B<sub>2</sub>, B<sub>6</sub> and *p*-aminobenzoic acid, 0.5; pantothenic acid and nicotinic acid amide, 2; inositol, 4; biotin, 0.0025.

Bacterial plasmoptyzate was tested for its ability to replace p.y.j. in the growth of *Paramecium*. When medium which gives good growth of *Colpoda*—1 part of plasmoptyzate to 4 parts of 5% brewers' yeast extract—was tried, the paramecia died after about three fissions in each trial. Other experiments using p.y.j. have indicated that this concentration of brewers' yeast extract is not favorable for *Paramecium*. However, very good growth of *Paramecium* occurred in a medium containing 4 cc. of 0.5% Difco yeast-extract, 1 cc. of plasmoptyzate and 0.5 cc. of 5% brewers' yeast-extract. Heated plasmoptyzate was inactive for *Paramecium* in this medium.

Pressed yeast juice was then tested for its ability to replace plasmoptyzate in the growth of *Colpoda*. A number of p.y.j. preparations as well as several fractions of p.y.j. which were active for *Paramecium* were tested in medium containing proteose-peptone as used for *Paramecium*; in medium containing brewers' yeast-extract; in medium containing Difco yeast-extract with a mixture of vitamins; and in dilutions of from 1 : 2.5 to 1 : 10,000 in Osterhout's balanced salt medium. No significant growth of *Colpoda* was obtained in any of these tests. It seems likely that the Fleischmann's yeast used for the preparation of p.y.j. contained the heat-labile substance essential for *Colpoda*. This ciliate has been successfully cultured on *S. exiguis* (Tatum *et al.*, 1942), and in direct tests *Colpoda* and *Paramecium* both grew well in media containing cells of *S. cerevisiae* (8.1.2.)<sup>4</sup> which had been repeatedly frozen and thawed.

#### DISCUSSION

The evidence suggests that p.y.j. supplies two fractions which are essential for the growth of *Paramecium*. One is heat-stable and the other heat-labile, and apparently protein in nature. Not only is it heat-labile and non-dialyzable, but it can be precipitated repeatedly with ammonium sulfate without loss of activity. The active substance must be either a protein or a substance so intimately bound to a protein that it cannot be removed by repeated precipitation of the protein nor by exhaustive dialysis. The loss of activity of p.y.j. on heating at 55°C. for thirty minutes and the partial recovery of activity after two weeks at 10°C. suggests the reversible heat denaturation of an active protein (see Neurath *et al.*, 1944).

<sup>4</sup>The authors wish to thank Dr. C. B. van Niel for supplying this strain, which was isolated in 1937 from Fleischmann's bakers' yeast.

On the basis of the experiments reported in this paper, *Colpoda* and *Paramecium* may require different heat-labile factors, both of which are present in plasmoptyzate, and only one present in our p.y.j. Alternatively, if the two ciliates require a common factor, this may be present in a filterable form in our p.y.j., apparently inactive for *Colpoda*, and in a non-filterable form in plasmoptyzate and probably also in yeast with activity for both *Colpoda* and *Paramecium*. The latter hypothesis is supported by our findings that the substance in plasmoptyzate which has activity for *Paramecium* does not pass through a Seitz filter. In this respect, therefore, the active substance in plasmoptyzate seems to differ from the active fraction in p.y.j.

The authors wish to acknowledge their indebtedness to Dr. Laura Garnjobst for preparing the bacterial plasmoptyzate samples, and for carrying out the tests with *Colpoda*.

#### SUMMARY

Pressed yeast juice contains two fractions essential for the growth of *Paramecium multimicronucleata*: (1) a heat-labile, non-dialyzable fraction which can be precipitated with ammonium sulfate, and (2) a heat-stable dialyzable fraction.

The heat-labile fraction of pressed yeast juice can be replaced by bacterial plasmoptyzate.

The possible protein nature of the heat-labile fraction of pressed yeast juice is discussed.

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# Amino Acid Interrelationships in the Nutrition of Proteus Morganii

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Received August 1, 1945

## INTRODUCTION

Several investigators have observed that certain amino acids in relatively high concentrations are toxic for bacteria.

For example, Kobayashi and Nishikawa (1921), Wyon and McLeod (1923), and Gordon and McLeod (1923) reported that glycine, phenylalanine, and tyrosine were inhibitory to the growth of various bacterial species in concentrations from 0.1 to 1.0%. More recently, Snell and Guirard (1943) have found that glycine, threonine, serine and  $\beta$ -alanine inhibit the growth of *Streptococcus fecalis* at high levels, but that the action of each of these substances is counteracted by the addition of more pyridoxine or alanine to the medium. Likewise, Fox, Fling, and Bollenback (1944) have observed that the development of *Lactobacillus arabinosus* in a chemically-defined medium is inhibited by *d*-leucine; they believed that in this case the *d*-form interferes with the metabolism of the normal *l*-amino acid.

Since 1939 it has also become apparent that certain of the amino acids are inhibitory under various conditions for some bacteria at low concentrations, and that this inhibition can be neutralized by other amino acids. Gladstone (1939) found that when isoleucine (in a concentration of  $M/312,500$ ), valine ( $M/187,000$ ), or leucine ( $M/42,500$ ) was added separately to a medium of known composition capable of supporting the growth of *Bacillus anthracis*, multiplication was prevented. The "toxic" effect of valine could be counteracted by leucine and *vice versa*, but that of isoleucine could only be "neutralized" by the presence of both valine and leucine. When all three of

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\* We wish to acknowledge and thank Merck & Co. for generously supplying the vitamins and several of the amino acids employed in this study; also Dr. V. du Vigneaud of Cornell University Medical College for most of the sulfur compounds tested, Dr. Richard J. Block, Scarsdale, N. Y., for the zinc hydroxy-desamino-methionine, Drs. W. C. Rose and Madelyn Womack of the University of Illinois for the *d*-methionine, Dr. J. O. Lampen of the American Cyanamid Co. in Stamford, Conn., for the methionine, and Dr. H. L. Fisher of U. S. Industrial Chemicals Research Laboratory, Stamford, Conn., for a sample of highest purity methionine.

these amino acids were added together, growth was improved and accelerated. Similar interrelationships were demonstrated with other amino acids. Harris and Kohn (1941) also noted that ethionine, norleucine and norvaline in concentrations of  $10^{-3} M$  to  $10^{-4} M$  inhibited the growth of a strain of *Escherichia coli* to the extent of 50 to 85% when added separately to simple media. This inhibitory action could be neutralized by the addition of other amino acids, including methionine. Likewise, Pelczar and Porter (1943) observed that when  $M/1500$  concentrations of allothreonine, norleucine and norvaline were incorporated separately in a chemically-defined medium they prevented the development of *Proteus morganii*. However, when these compounds were present in a mixture of several amino acids no inhibition occurred.

The occurrence of amino acid interrelationships in the nutrition of other microorganisms has also been noted. For example, Nielsen and Hartelius (1938) showed that  $\beta$ -alanine exerts a toxic effect on yeast unless asparagine or aspartic acid is added to the medium; Doermann (1944) found that certain concentrations of arginine specifically inhibited the growth of a mutant of *Neurospora crassa* which required lysine; and Kidder and Dewey (1945) have observed that several amino acids are "toxic" for the ciliate *Tetrahymena*, and that the inhibition may be released by certain other amino acids.

The present study was thus undertaken to determine what amino acids neutralize the toxicity of allothreonine, norleucine and norvaline for *Proteus morganii*.

### TECHNIQUE

The strains of *Proteus morganii* used and the other technical details followed in this study were the same as reported in previous papers from this laboratory (see Pelczar and Porter, 1943; Meyers and Porter, 1945). The basal medium employed had the following composition:

$\text{NH}_4\text{Cl}$	1.0 g.
$(\text{NH}_4)_2\text{SO}_4$	1.0 g.
$\text{NaCl}$	1.0 g.
$\text{KH}_2\text{PO}_4$	1.0 g.
$\text{K}_2\text{HPO}_4$	1.0 g.
$\text{MgSO}_4$	0.1 g.
Distilled water to 950 ml.	
pH adjusted to 7.2-7.4 with $N/\text{NaOH}$	

This medium was tubed in 9.5 ml. amounts, autoclaved and then fortified with the following:

	Final Concentration
Glucose (0.1 ml. of sterile stock solution)	0.5%
<i>l</i> -cystine (0.1 ml. of sterile stock solution)	$24.0 \gamma/\text{ml.}$
Ca-pantothenate (0.1 ml. of sterile stock solution)	$1.0 \gamma/\text{ml.}$
Nicotinamide (0.1 ml. of sterile stock solution)	$1.0 \gamma/\text{ml.}$
Sterile distilled water to make 10 ml.	

The growth factors and amino acids were prepared in concentrated solutions and filtered through sintered glass filters for sterilization. The stock solution of glucose (50%) was autoclaved.

The quaternary sulphonium-methionine compound used in this study was prepared by the method of Toennies (1940), and the  $\alpha$ -keto acid analogue of methionine was synthesized following the procedure described by Cahill and Rudolph (1942).

### EXPERIMENTAL AND RESULTS

The data in Table I show the molar concentrations of allothreonine, norleucine and norvaline which are inhibitory when these amino acids

TABLE I

*Inhibitory Action of Allothreonine, Norvaline and Norleucine for Proteus morganii*

Concentration of amino acid added to the basal medium		Growth of Strains of <i>Proteus morganii</i> at Various Times (days)																	
		P-3*			P-10			P-15			P-18			P-21			P-24		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Allothreonine	M/1500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M/3000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M/6000	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
	M/750,000	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Norleucine	M/1500	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M/3000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	M/6000	-	-	-	-	?	+	?	+	+	-	-	+	-	?	+	-	?	-
	M/750,000	-	-	-	-	+	+	+	+	+	-	?	+	-	+	+	+	+	?
	M/1,500,000	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	+
Norvaline	M/1500	-	?	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	+
	M/3000	-	+	+	-	+	+	+	+	+	+	+	+	-	-	-	-	+	+
	M/6000	+	+	+	+	+	+	+	+	+	+	+	+	-	?	+	+	+	+
	M/750,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Basal medium (control)		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = good growth; - = no growth; ? = questionable growth

\* refers to culture number

are added separately to the basal medium. The results disclose that allothreonine at a concentration above M/3000 inhibits the growth of the six strains of *Proteus morganii* tested, whereas M/6000 is not toxic except for one strain. Norleucine exhibited some action at concentrations as low as M/750,000 to M/1,500,000 and usually prevented the growth of all of the strains employed for at least three days at concentrations higher than M/3000. Norvaline was the least toxic of the three amino acids, since M/1500 to M/3000 concentrations permitted many of the strains to grow within three days. In most cases, when the tubes

containing the inhibitory concentrations of the amino acids were incubated for four to six days some growth finally occurred.

Twenty-nine amino acids and related compounds were then tested separately to ascertain their ability to "neutralize" the inhibitory effect of allothreonine, norleucine and norvaline for *Proteus morganii*. The results of these tests are summarized in Table II. Some twenty

TABLE II

*Amino Acids and Derivatives (M/1500) which Reversed the Inhibitory Action of Allothreonine (M/750), Norleucine (M/1500) and Norvaline (M/750) for Proteus morganii (7 strains) in 1-7 Days*

Amino Acid	Allothreonine	Norleucine	Norvaline
<i>dl</i> -alanine	+	-	-
allothreonine		-	+?
<i>d</i> -arginine·HCl	+	-	-
<i>l</i> -asparagine	*	-	*
<i>dl</i> -aspartic acid	*	-	*
<i>dl</i> -citrulline	*	-	*
<i>l</i> -cystine	-	-	-
glycine	+	-	+?
<i>dl</i> -glutamic acid	++	-	-
hippuric acid	*	-	*
<i>l</i> -histidine·HCl	+	-	-
<i>l</i> -hydroxyproline	+	-	-
iodogorgoic acid	+?	-	-
<i>dl</i> -isoleucine	+	-	-
<i>dl</i> -leucine	+	**	+
<i>dl</i> -lysine·HCl	+	-	-
<i>dl</i> -methionine	+	+++	+?
methoxinine	-	-	-
<i>dl</i> -norleucine	-		-
<i>dl</i> -norvaline	+?	-	-
<i>d</i> -ornithine·2HCl	+	-	-
<i>dl</i> -phenylalanine	+	-	-
<i>l</i> -proline	+	-	-
<i>dl</i> -serine	+++	-	-
taurine	+	-	-
<i>dl</i> -threonine	+	-	-
<i>l</i> -tryptophane	++	-	-
<i>dl</i> -tyrosine	+	-	-
<i>dl</i> -valine	++	-	+

- = no growth

+ to +++ = degree of growth

\* = not tested

\*\* = leucine (M/750) reversed the inhibition with one strain

amino acids were capable of counteracting the action of allothreonine, while four reversed the toxicity of norvaline, and only one (methionine) consistently "neutralized" norleucine when used in low concentrations.

Since the toxic action of norleucine for *Proteus morganii* was more pronounced than the inhibition exhibited by allothreonine and norvaline, and since its neutralization was more specific, it was decided to study this amino acid interrelationship in more detail. Table III shows,

TABLE III

*Concentration of Methionine Required to Neutralize the Inhibitory Action of Various Concentrations of Norleucine for Proteus morganii (7 strains)*

Basal medium plus	Norleucine Concentration and Growth in Days									
	M/1500			M/750			M/375			
	1	2	3	1	2	3	1	2	3	
<i>M/2000 to M/80,000</i>	+	+	+	+	+	+	+	+	+	
<i>M/200,000</i>	+	+	+	+	+	+	-	+	+	
<i>M/400,000</i>	+	+	+	+	+	+	-	+	+	
<i>M/800,000</i>	+	+	+	-	+	+	-	+?	+	
<i>dl</i> -Methionine	<i>M/1,500,000</i>	-	+	+	-	+	+	-	+?	+
	<i>M/2,000,000</i>	-	+	+	-	+	+	-	+?	+
	<i>M/4,000,000</i>	-	+?	+	-	-	+	-	-	+
	<i>M/8,000,000</i>	-	+?	+	-	-	+	-	-	+?
	None (control)	-	-	+?	-	-	-	-	-	-

+= good growth; +? = questionable growth, or variable with different strains;

- = no growth

for example, that the three concentrations (*M/1500*, *M/750*, *M/375*) of norleucine inhibit the growth of *Proteus morganii* for at least one day in the presence of *M/1,500,000*, *M/800,000* and *M/200,000* methionine, respectively. Higher concentrations of methionine than these neutralized the action of norleucine and growth occurred within one day.

Since methionine was the only one of the more common amino acids which would consistently reverse the toxic action of norleucine, several other sulfur-containing compounds and substances related to methionine were tested to determine their ability to antagonize the action of norleucine. The results of a few of these tests are shown in Table IV. Although lanthionine allowed slight neutralization, all of the other

TABLE IV

*Ability of Various Sulfur-Containing Compounds to Replace Methionine in the Methionine in the Neutralization of the Inhibitory Action of Norleucine for *Proteus morganii* (6 strains)*

Basal medium plus norleucine ( <i>M</i> /750) plus	Quantity $\gamma/ml$	Growth of <i>Proteus morganii</i> in Days			
		1	2	3	4
<i>L</i> -cystine	120	—	—	—	—
	240	—	—	—	—
	480	—	—	—	—
*cystine disulfoxide	24	—	—	—	+?
*lanthionine	24	+	+	+	+
	6	—	—	—	+
*djenkolic acid	24	—	—	—	—
*cystathionine	24	—	—	—	—
<i>dl</i> -methionine	24	+++	++++	++++	++++
<i>d</i> -methionine	24	+++	++++	++++	++++
methionine-methyl-sulfonium iodide	24	++	++	++	++
Zn-hydroxy-desamino-methionine	24	++	++	++	++
$\gamma$ -keto acid analogue of methionine	24	++	++	++	++
methyl-N-methionine	24	—	—	—	—
ethionine	24	—	—	—	—
* <i>L</i> -homocystine	24	—	—	—	—
* <i>dl</i> -homocysteine	24	—	—	—	—
pentocystine	24	—	—	—	—
hexocystine	24	—	—	—	—
*thioacetamide	24	—	—	—	—
*Na <sub>2</sub> S	24	—	—	—	—
*Na <sub>2</sub> S <sub>2</sub>	24	—	—	—	—

\* Compounds which will replace cystine completely or partially in the nutrition of *Proteus morganii* (see Meyers and Porter, 1945).

compounds, with the exception of some of the methionine derivatives, were completely negative. It will also be seen from the data in the table that both the *dl*- and the *d*- forms of methionine are active, as well as methionine-methyl-sulfonium iodide, the  $\alpha$ -keto acid analogue of methionine, and zinc hydroxy-desamino-methionine. It is of interest that methyl-N-methionine, ethionine, and methoxinine are inactive in so far as the neutralization of norleucine is concerned. It is known, however, that these latter two compounds are inhibitory for certain

bacteria (see Harris and Kohn 1941; Roblin, Lampen, English, Cole, and Vaughn 1945).

It is not known yet why norleucine exhibits a toxic effect on *Proteus morganii* or the mechanism whereby methionine neutralizes this action. It was thought that norleucine may be interfering with the ability of methionine to function in transmethylation. However, since studies in which various concentrations of homocysteine or homocystine and choline were substituted for methionine were negative, this does not appear to be an explanation. The only other data which we have at present concerning the interrelationship between norleucine and methionine, are based on the production of H<sub>2</sub>S from cystine by this organism. Using the "aeration train" technique of Tarr (1933) it was found that when a suspension of washed cells was suspended in a buffered-cystine solution they produced 2730 micromilligrams of H<sub>2</sub>S from 120 mg. of cystine. However, when norleucine was added to another flask, not enough H<sub>2</sub>S was produced to be determined by the method of Almy (1925). This phase of the work is being continued and we hope to present more positive data on this phenomenon at a later date.

#### SUMMARY

Certain concentrations of allothreonine, norleucine, and norvaline have been shown to inhibit the growth of *Proteus morganii* in a chemically-defined medium. Several amino acids will counteract the action of allothreonine and norvaline, but only methionine and certain of its derivatives will consistently reverse the "toxicity" of norleucine when present in small amounts.

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# The Action of Citrinin on Some Respiratory Enzymes of *Staphylococcus Aureus* and *Escherichia Coli*<sup>1</sup>

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Received August 1, 1945

## INTRODUCTION

Investigations of the effect of antibiotic substances upon bacteria have, to date, been largely concerned with the specificity and phase of development of the test organism, and with the environmental conditions of the medium that allow the occurrence of bacteriostasis.

The purpose of the experiments to be reported herein, was to gain some insight into the mechanism of action of an antibiotic during the susceptible phase of the organism.

Accordingly, an experiment was planned to determine the relative effect of an antibiotic on the enzymes of susceptible and non-susceptible bacterial species. This work was based on the concept of Green (5) that any organic substance which is physiologically active in extreme dilutions must be a component of an enzyme system or else it specifically interferes with some such system. Characteristically, antibiotics are effective at very low dilutions. Hence, if this concept be valid, it would follow that the site of action of antibiotic substances is likely to be associated with the activity of specific enzymes.

The reaction first investigated was the effect of an antibiotic on the lactate-oxidase and glucose-oxidase systems, these being systems common to bacterial species that are, respectively, susceptible and resistant to several antibiotics.

Citrinin was the antibiotic chosen for these experiments because: (a) its molecular structure was known (2, 6); (b) its yield from culture solutions is high (1, 10); (c) its antibiotic potentialities had been determined (9, 11); and (d) it is stable and resistant to autoclaving (11).

<sup>1</sup> Macdonald College, Journal Series No. 206.

## MATERIALS AND METHODS

*Bacteria:* *S. aureus* H was used as the species susceptible to citrinin, and *B. coli* the non-susceptible species.

The enzymes chosen for study were those most active in the two species, lactate oxidase being common to both.

The test specimens were prepared in the following manner: 10 ml. of 16 hr. cultures in nutrient broth, incubated at 37°C., were added to each of two 200 ml. lots of nutrient broth. This was incubated for 3 hrs. at 37°C., preliminary tests having indicated that after this period of incubation the bacteria exhibited the effects described later most distinctly. Finally, the cells were separated by centrifuging; washed in M/5 phosphate buffer at pH 6.8; recentrifuged and the total cell mass suspended in 10 ml. of the same buffer. This suspension contained from 8 to 12 × 10<sup>9</sup> cells per ml.

For use in Warburg vessels 0.6 ml. of this suspension was added as inoculum for each vessel, which contained a total volume of 2.5 ml. The centre well of each vessel contained 0.2 ml. of 50% KOH solution for absorption of carbon dioxide. Varying amounts of suspension were used in the Thunberg experiments.

*Citrinin:* The citrinin had been purified by precipitation from sodium bicarbonate solution with HCl, washed with water and dried. For use, it was dissolved in sodium citrate-sodium chloride solution (1.05% Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub> · 2H<sub>2</sub>O + 0.8% NaCl) to give 0.5 mg. per ml.

*Substrates:* The substrates used were lithium lactate 0.5%, \* M/10 sodium succinate and M/10 glucose.

*Equipment:* Respiratory tests were carried out with standard equipment for the Warburg and Thunberg techniques keeping the water-baths at 37°C.

For each Warburg test an equilibration time of 30 minutes was allowed prior to admixture of the citrinin and substrate.

*Bacterial Counts:* The numbers of bacteria introduced into the Warburg vessels and the numbers surviving at the conclusion of each experiment were determined by standard bacteriological plating procedures.

## EXPERIMENTAL

### *Experiment 1: The Influence of Citrinin on Oxygen Uptake by S. aureus and B. coli*

Table I records one of several typical experiments. Oxygen uptake is expressed in cubic millimeters after the stipulated time intervals. The results show that the oxygen uptake, with either lactate or glucose as donor, is depressed in the presence of citrinin. The decrease is manifest within 15 minutes after addition of citrinin to the mixture of bacteria and substrate. After 120 minutes an 80% inhibition is observed. The action of citrinin is thus seen to be almost immediate.

\* Courtesy of Foote Mineral Company, Philadelphia, Pa., U. S. A.

TABLE I

*Effect of Citrinin on Oxygen Uptake of S. aureus with Lactate and Glucose as Substrates*

Duration of exposure to citrinin mins.	Oxygen uptake in *			
	Lactate mm. <sup>3</sup>	Lactate and citrinin mm. <sup>3</sup>	Glucose mm. <sup>3</sup>	Glucose and citrinin mm. <sup>3</sup>
15	8.7	2.4	7.0	2.2
30	17.7	5.5	21.0	6.3
45	29.6	8.2	37.1	8.6
60	45.5	14.6	61.5	15.6
75	67.4	16.5	77.3	15.7
90	75.3	18.4	101.7	19.7
105	94.6	21.5	126.4	23.5
120	118.9	24.6	153.5	27.0

\* Average of 2 vessels.

In order to determine whether this decrease could have been caused by a reduction in bacterial numbers due to some bactericidal action, the numbers of bacteria remaining alive after successive experimental periods were determined. The numbers of surviving bacteria were extremely variable but showed no relation whatever to the oxygen uptake. Garrod (4) has made a similar observation regarding the number of bacteria after treatment with Ringer's solution and penicillin. Critical examination of all phases of the plating method indicated that during the conditions of the experiment, whether citrinin was present or not, the bacteria were dying rapidly. Hence, efforts were made to kill as many cells as possible in order to determine whether citrinin would inhibit oxygen uptake in preparations containing only dead cells. Accordingly, washed cells suspended in buffer were killed by alternate freezing and thawing carried out 12 times during a period of 6 hrs. The cells were then frozen overnight and used immediately after the final thawing. The freezing temperature was -20°C., the thawing temperature 25°C. Plate counts showed that the number of bacteria had been reduced from 12 billion to less than 100,000 per ml.

Data from this experiment are summarized in Table II which illustrates inhibition of oxygen uptake by citrinin in the presence of killed

TABLE II

*Effect of Citrinin on Oxygen Uptake of Suspensions of Cells of S. aureus Subjected to Alternate Freezing and Thawing*

Experiment number	Oxygen uptake in mm. <sup>3</sup> after 60 minutes	
	Lactate	Lactate + citrinin
1	53.2	38.9
2	55.1	37.2
3	49.8	33.6

cells. It is to be noted that inhibition by citrinin is less where killed cells predominate but is, nevertheless, quite definite.

Table III demonstrates the effect of increasing concentrations of citrinin on oxygen uptake. The increase in inhibition is seen to parallel increase in citrinin concentration.

TABLE III  
*Effect of Varying Concentrations of Citrinin on Oxygen Uptake by  
S. aureus Using Lactate as Donor*

Concentration of citrinin mg. per cent	Oxygen uptake in mm. <sup>3</sup> after 60 minutes		
	Exp. 1	Exp. 2	Exp. 3
0.0	104.6	85.2	76.0
2.0	91.5	63.0	69.9
6.0	84.8	51.0	57.3
10.0	68.6	35.6	52.8

A similar experiment carried out with *B. coli* and summarized in Table IV indicates that the overall oxygen uptake by this organism is

TABLE IV  
*Influence of Citrinin on the Oxygen Uptake of B. coli*

Donor *	Oxygen uptake in mm. <sup>3</sup> after 60 minutes
Succinate	208.7
Succinate + citrinin	208.4
Lactate	111.0
Lactate + citrinin	99.4

\* Different bacterial suspensions used with each donor.

impaired by citrinin to only a slight degree. This would be in agreement with the observation by Timonin (11) that growth of *B. coli* is only slightly suppressed by this antibacterial substance.

*Experiment 2: The Influence of Citrinin on the Dehydrogenases of Bacteria Susceptible and Nonsusceptible to Citrinin Bacteriostasis—Thunberg Method*

The respective rates of decolorization of methylene blue as brought about by *S. aureus* and *B. coli*, each with and without citrinin, are indicated in Table V.

It can be seen that in the presence or absence of citrinin, the bacterial suspensions used had approximately the same dehydrogenase activities.

TABLE V

*Influence of Citrinin on Decolorization of Methylene Blue by S. aureus and B. coli—Thunberg Method*

Exp. No.	1	2	3	4	5	6	7	8
<i>S. aureus</i> (ml. of suspension)	0.3	0.3	0.3	—	—	—	—	—
<i>B. coli</i> (ml. of suspension)	—	—	—	0.3	0.3	0.3	0.3	0.3
Lactate (ml. of 1% solution)	0.5	0.5	0.5	—	0.5	0.5	0.5	0.5
Citrinin (mg. per cent)	0.0	5	2.5	0.0	—	5.0	2.5	0.5
* Time of decolorization of M.B. (minutes)	8	18	9½	45	10	11	11	10

\* 0.5 ml. of 1 : 5000 M.B. added to a total volume of 5 ml.

Hence, it is clear that citrinin, with lactate as hydrogen-donor, impairs the decolorization rate of the citrinin-sensitive *S. aureus* to a marked degree, while the non-sensitive species, *B. coli*, is scarcely influenced at all. The effective concentration of citrinin for inhibition of the lactate dehydrogenase system of *S. aureus*, with the numbers of bacteria used, was 2.5 mg. per cent.

### DISCUSSION

The experiments concerning oxygen uptake and hydrogen transfer show that certain enzymes of *S. aureus* H are inhibited by citrinin. The enzymes of *B. coli*, which is non-susceptible to bacteriostasis by citrinin, are not inhibited. Hence, a positive correlation exists in this instance between inhibition of enzymes and bacteriostasis. It is suggested that this correlation gives at least a partial insight into the mechanism of bacteriostatic action. Hotchkiss (8) in a reference to unpublished work, makes a similar inference with respect to gramicidin. No data were presented. Dorfman *et al.* (3) find an analogous differential reaction between susceptible and nonsusceptible species with respect to modification of surface charge induced by penicillin. This latter observation is interpreted as a possible influence on cell-permeability which, however, could not be the sole explanation of bacteriostasis with respect to citrinin in this instance, for inhibition of enzymic action is noted even with suspensions of dead cells the protoplasmic surfaces of which would be destroyed.

The authors wish to express their thanks to Dr. M. I. Timonin, Division of Bacteriology and Dairy Research Service, Dept. of Agriculture, Ottawa, Canada, for the citrinin used in these experiments, and for his sustained interest in this work.

## SUMMARY

The antibiotic substance, citrinin, inhibits certain respiratory enzymes in *S. aureus*, which is susceptible to citrinin, but does not inhibit the same enzymes in the resistant species, *B. coli*.

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# The Fermentation of Glucose by *Clostridium tetani*

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Received August 1, 1945

## INTRODUCTION

Although glucose is routinely added to the broth media used in this laboratory for carrying cultures of *Clostridium tetani*, we have attempted, without success, to demonstrate a utilization of this sugar during growth of the bacillus. In the course of this study Reed and Orr (1943) presented data which indicated that glucose may serve only to lower the potential of the medium to a level suitable for growth of these bacteria. Nevertheless, our data lead us to believe that glucose may actually be fermented slowly by "normal" strains of *Cl. tetani*.

More recently, our attention has been directed to a "mutant," glucose-fermenting strain of the tetanus bacillus. This strain is of considerable interest, not only because of its ability to ferment glucose but also because of the parallelism between this fermentation and the concentration of iron in the growth medium. Furthermore, it has been shown (Mueller and Miller, 1945) that this strain yields a toxic filtrate of unusually high titer.

The following report is concerned with the fermentation of glucose by a "mutant" or "trained" strain—referred to in this paper as the "TA" strain—of *Cl. tetani*, and with preliminary observations on the role of iron in the fermentation of this sugar by the same strain of the bacillus.

## EXPERIMENTAL

### Methods

Determinations of acetone, alcohol, reducing sugar, volatile acids, and carbon dioxide were carried out according to the methods previously employed in this laboratory (Pickett, 1943; Pickett *et al.*, 1944). Glycerol was determined according to Voris *et al.* (1940), and lactic acid was determined according to the method of

Barker and Summerson (1941). Iron was determined spectrophotometrically using  $\alpha,\alpha'$ -dipyridyl, in a modification of the method of Koenig and Johnson (1942).

Tryptically digested meat infusion broth was prepared in the conventional fashion. Hog stomach medium enriched with 0.75% glucose was prepared according to Mueller and Miller (1945). Iron was removed from the medium by adsorption on calcium phosphate (Pappenheimer and Johnson, 1936).

Washed suspensions of the organisms were obtained from 18-hour cultures which were grown in deep vessels without anaerobic precautions at 35° C. The cultures were then harvested by centrifugation at 2000 r.p.m. for 60 minutes, suspended in de-oxygenated physiological saline solution, and recentrifuged at 2500 r.p.m. for 15 minutes. The cells were then resuspended in de-oxygenated saline solution and used immediately in the Warburg vessels.

### Results

#### 1. Fermentation of Glucose by Washed Suspensions from Iron-Rich Media

After it was established that the mutant "TA" strain of *Cl. tetani* could utilize glucose, an attempt was made to determine quantitatively the products of the fermentation. In order that glucose might be used as a substrate and the complicated breakdown products of a natural growth medium still be avoided, washed suspensions of the organisms were used. All the organisms used in this series of experiments were obtained from cultures grown in the hog stomach medium of Mueller and Miller (1945). This medium was enriched with 250–300 γ of reduced iron per ml. in the form of powdered iron. Results of Warburg experiments gave preliminary indications that the fermentation of glucose by *Cl. tetani* was essentially alcoholic, approximately two moles of CO<sub>2</sub> and two moles of alcohol being formed per mole of sugar fermented (Table I). The amount of CO<sub>2</sub> produced varied from 94% to 107% of the theoretical two moles of CO<sub>2</sub> per mole of glucose.

The amount of alcohol produced was approximately that for the theoretical two moles of alcohol per mole of glucose. Approximately one mole of H<sub>2</sub> was formed per ten moles of glucose fermented.

In one Warburg experiment, determination was carried out for lactic acid but none was found. Later, determinations for lactic acid carried out on the products of larger scale fermentations indicated that this compound was present in small amounts.

The utilization of glucose substrate by washed suspensions of the "TA" strain of *Cl. tetani* was then carried out on a larger scale in an attempt to obtain a more complete fermentation balance. Krebs's fermentation flasks of approximately 300 ml. capacity were employed for

TABLE I

*Fermentation of Glucose by Washed Suspensions of *Cl. tetani**

Experiment No.	Duration of Fermentations: 3 to 5 hours		
	1	2	3
Micromoles glucose added	10.0	5.0	5.0
Micromoles H <sub>2</sub> found	1.1	0.0	0.5
Micromoles CO <sub>2</sub> found	19.4	10.7	9.4
Micromoles alcohol found	20.2	— <sup>1</sup>	8.8
Micromoles lactic acid found	none	— <sup>1</sup>	0.1

<sup>1</sup> Not determined.

these experiments. Fifty ml. of washed, buffered, bacterial suspension and one millimole of glucose were added to the flasks in an atmosphere of pre-purified N<sub>2</sub>, and these were incubated for 48 hours at 35°C. Qualitative determinations were carried out for various end-products of carbohydrate metabolism, and if any of these was found to be present it was measured quantitatively. The results are summarized in Table II. More than 99% of the sugar was fermented, the chief end-products being CO<sub>2</sub> and alcohol. Approximately 1.7 moles of CO<sub>2</sub> and 1.2 moles of alcohol were formed per mole of glucose fermented. The alcohol was tentatively identified by dichromate oxidation followed by ether partition and Duclaux distillation of the corresponding acids. The alcohol appeared to be 88% ethyl and 12% butyl. Less than two micromoles of lactic acid were formed per mole of glucose fermented, and only a trace of acetone was found. These end-products accounted for only 74% of the hexose carbon which was fermented.

## 2. Relationship of Iron Concentration to Activity of the Organisms

The relationship of the concentration of iron in the medium to the glucose-fermenting activity of washed suspensions of the "TA" strain of *Cl. tetani* was investigated.

For these studies, hog stomach medium was prepared so as to be essentially iron-free. Analyses of this medium after the removal of iron showed that the maximum amount remaining was 0.02 γ of iron per ml. of medium. All glassware used in this work was cleaned routinely in sulphuric acid-potassium dichromate mixture, then in concentrated hydrochloric acid to remove possible traces of iron. It was then subjected to several rinsings with tap water, distilled water, and finally doubly distilled water. Varying concentrations of inorganic iron in the form of sterile FeSO<sub>4</sub>·7H<sub>2</sub>O solution were added to the media before these were inoculated. The inocula were obtained from a vigorously fermenting 12-hour broth culture, the amounts being 2.5% of the

TABLE II

*Balance-sheet for Fermentation of Glucose by *Clostridium tetani**

Substrate, 20.0 ml. 0.05 *M* glucose; washed suspension of cells, 10 ml. (20.9 mg./ml. dry weight); phosphate buffer (0.05 *M*), pH 7.0; temp., 35°C.; total volume, 50.0 ml.; time, 48 hours; atmosphere, N<sub>2</sub>.

Products found	Moles/100 moles	C atoms/6 C atoms glucose
CO <sub>2</sub>	165.0	1.65
Ethyl alcohol	104.0	2.08
Butyl alcohol	14.8	0.592
Volatile acid <sup>1</sup>	1.65	0.033
Lactic acid	0.16	0.005
Acetone	Trace	—
Glycerol	None	—
Glucose unfermented	1.0	0.06
Total		4.420 <sup>2</sup>

<sup>1</sup> Volatile acid is assumed to be a 2-carbon acid in the carbon balance.

<sup>2</sup> 4.42 C atoms = 74% theoretical yield.

volume of the inoculated media. These cultures were incubated, harvested, and re-suspended as described above. A small aliquot of each suspension was saved for turbidity reading with the Coleman spectrophotometer, in order to determine the dry weight of cells used in each vessel. A typical protocol for these experiments is given in Table III, and the results are shown graphically in Figs. 1 and 2.

It was found that the concentration of iron in the medium had a direct effect on the glucose-fermenting activity of the organisms. Organisms grown in the absence of iron had practically no activity, and, as increasing amounts of iron were added to the medium the activity of the suspensions progressively increased. The  $\mu\text{L}$  of CO<sub>2</sub> produced per mg. dry weight of bacteria per unit time was found to increase in amounts corresponding to the increase of the iron concentration. The figures obtained for Q<sub>CO<sub>2</sub></sub> with iron concentrations of 10 and 100  $\gamma$  per ml. were irregular and somewhat lower than would be expected in the progressive increase of activity, and this was ascribed to the fact that the medium was blackened by iron reduction products during fermentation in the higher iron concentrations. These blackened precipitates could not be totally eliminated during the process of washing the cells, since excessively vigorous and prolonged handling of the organisms tended to destroy their activity. The presence of these precipitates during the spectrophotometric determination of turbidity would tend to give a falsely high value for the total dry weight of organisms, and a subse-

quently falsely low value for the activity of the suspensions which contained these precipitates. Fortunately, this difficulty was encountered only in the two highest iron concentrations mentioned above.

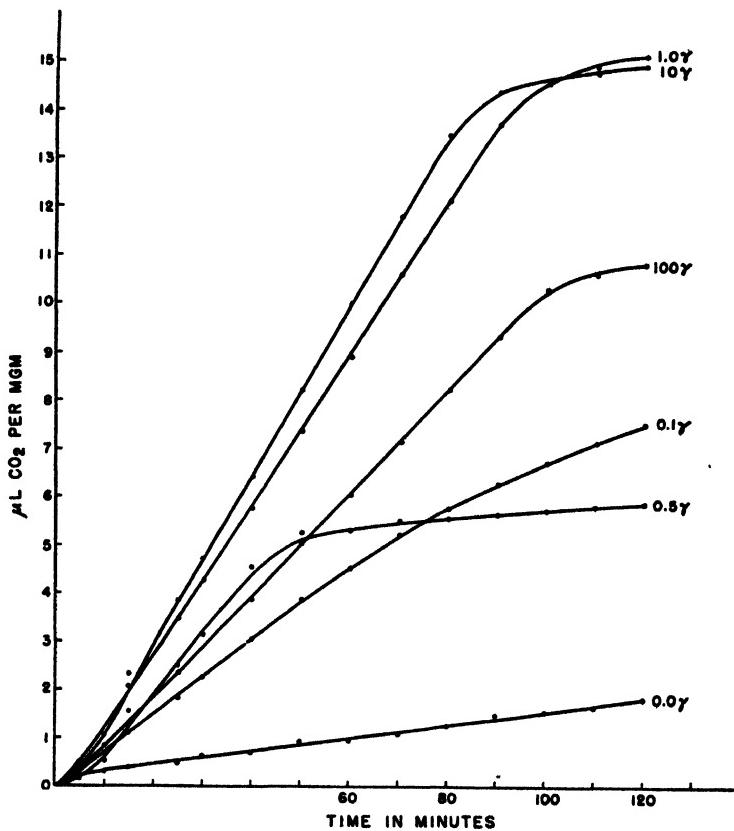


FIG. 1

Glucose-Fermenting Activity of *Clostridium tetani* at Various Iron Concentrations  
Figures at right of curves represent amount of iron per ml. of growth medium.

### 3. Stimulation of Glucose and Pyruvate Fermentation

Since iron was found to play an essential role in the fermentation of glucose by the "TA" strain of *Cl. tetani*, further studies were directed toward a more exact determination of its action in this process. These included, first, several manometric experiments in which inorganic iron

or boiled suspensions were added to the Warburg vessels in addition to glucose and buffered, active suspensions of washed cells. The few results obtained from suspensions with added iron (ferrous sulfate) were rather variable. With cells from a high iron medium, addition of inorganic iron did not appear to accelerate the fermentation of glucose. However, a slight stimulation of fermentation was obtained with cells grown in a low iron medium. In one experiment, e.g., a  $Q_{CO_2}$  of 2.0 was

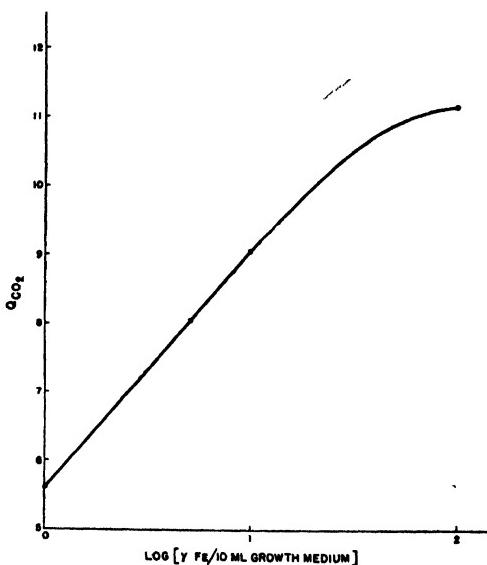


FIG. 2

Relationship of Glucose-Fermenting Activity of *Clostridium tetani* to Concentration of Iron in Growth Medium

obtained in the absence, and 2.8 in the presence, of 40  $\gamma$  of iron. In contrast, however, the  $Q_{CO_2}$  with cells from a high iron medium was 8.6.

A more definite stimulation of fermentation was obtained with boiled cells (Table IV). This stimulation was obtained with boiled cells from both high iron and low iron media and is, therefore, apparently not ascribable solely to an iron-containing enzyme. The possibility exists, however, that the boiled cells from low iron media may have elaborated small amounts of an iron-containing coenzyme from minute amounts of iron present in these media (0.02  $\gamma$ /ml., or less).

TABLE III

*The Effect of Iron on the Fermentation of Glucose by Clostridium tetani*

Iron added to growth medium, γ/ml.	0.0	0.1	0.5	1.0	10	100
Vessel No.	1	2	3	4	5	6
Additions to vessels:						
Washed suspension, ml.	1.0	1.0	1.0	1.0	1.0	1.0
Phosphate buffer, 0.02 M, pH 7, ml.	0.5	0.5	0.5	0.5	0.5	0.5
Glucose, 0.1 M, ml.	0.1	0.1	0.1	0.1	0.1	0.1
Water, ml.	0.5	0.5	0.5	0.5	0.5	0.5
Dry weight bacteria per vessel, mg.	19.3	43.0	76.0	27.0	27.8	36.8
Total CO <sub>2</sub> formed, μL	35	323	454	412	415	400
Q <sub>CO<sub>2</sub></sub> <sup>1</sup>	0.8	5.6	8.0	9.0	11.1	6.5

<sup>1</sup> Read from curves.

TABLE IV

*The Effect of Boiled Suspensions on the Fermentation of Glucose and Pyruvate by Washed Suspensions of Cl. tetani*

Experiment number	Iron added to growth medium (γ/ml.)	Additions to buffered suspensions in vessels		Per cent stimulation
		Substrate	Stimulant	
1	10	Glucose	Boiled high iron suspension <sup>1,4</sup>	24
1	10	Glucose	Boiled high iron suspension <sup>1,3</sup>	17
2	0	Glucose	Boiled high iron suspension <sup>1,3</sup>	20
2	0	Glucose	Boiled low iron suspension <sup>2,3</sup>	14
2	10	Glucose	Boiled high iron suspension <sup>1,3</sup>	20
2	10	Glucose	Boiled low iron suspension <sup>2,3</sup>	18
3	0	Glucose	Boiled high iron suspension <sup>1,3</sup>	42
3	0	Glucose	Boiled low iron suspension <sup>2,3</sup>	83
3	10	Glucose	Boiled low iron suspension <sup>2,3</sup>	14
4	0	Pyruvate	Boiled high iron suspension <sup>1,3</sup>	1
4	10	Pyruvate	Boiled high iron suspension <sup>1,3</sup>	5

<sup>1</sup> Boiled suspension of bacteria from a medium containing 10 γ iron/ml.<sup>2</sup> Boiled suspension of bacteria from a medium containing 0.02 γ iron/ml., or less.<sup>3</sup> Suspension boiled ten minutes.<sup>4</sup> Suspension boiled one minute.

#### 4. Effect of Enzyme Poisons on the Fermentation of Glucose and Intermediate Compounds by Washed Suspensions

The end-products obtained from the degradation of glucose by the "TA" strain of *Cl. tetani* were those of an alcoholic fermentation but, at the same time, the iron requirement for this fermentation indicated the presence here of a distinctly different metabolic pattern. A clue to the enzymes involved in this fermentation was sought by the use, in manometric experiments with washed cells, of several relatively specific poisons. In most of these experiments the washed cells were again obtained from 18-hour cultures grown in hog stomach medium. In a few experiments (Nos. 3-6, Table V) the cells were obtained from 18-hour cultures grown in tryptic digest medium. Reduced iron was added to both media to give approximately 10 γ of iron per ml. of medium. Higher concentrations of iron in the media did not increase the activity of the washed cells. The cells obtained from hog stomach medium had, in general, a greater activity than those grown in tryptic digest medium, but both were quite satisfactory for the examination of enzyme inhibition.

The inocula were obtained from a vigorously fermenting 24-hour broth culture, the amounts being 1-2% of the volume of the inoculated media. These cultures were incubated, harvested, and resuspended as described in the preceding sections.

In the first series of experiments (Table V), in which glucose was the fermentable substrate, it was found that monoiodoacetic acid in concentrations as low as  $M/10,000$  caused complete inhibition of fermentation. Sodium azide had no inhibitory effect in concentrations varying from  $M/1,000$  to  $M/100$  in one experiment. In another experiment this compound gave 8% inhibition in  $M/100$  and 1% inhibition in  $M/200$  concentration. Potassium arsenite gave inhibition ranging from 100% in  $M/50$  to 86% in  $M/1,000$  concentration. Potassium cyanide gave 58% inhibition in  $M/50$  and 33-35% inhibition in  $M/100$  concentration. In lower concentrations of cyanide ( $M/200-M/5,000$ ), inhibition varied from 20 to 11%.

One hundred per cent carbon monoxide gave 0 to 2% inhibition, and in lower concentrations gave no inhibition. In one experiment (No. 14) in which 100% carbon monoxide was used as an inhibitor, the first part of the fermentation was conducted in the dark. Subsequent exposure to intense light for a period of 90 minutes had neither a stimulatory nor an inhibitory effect on the fermentation.

TABLE V

*The Effect of Selective Poisons on the Fermentation of Glucose by Cl. tetani*

Expt. No.	Poison	Concentration	Inhibition <sup>1</sup> per cent
5	Monoiodoacetate	<i>M</i> /1,000	100
5	Monoiodoacetate	<i>M</i> /10,000	100
3	Azide	<i>M</i> /100	0
19	Azide	<i>M</i> /100	8
19	Azide	<i>M</i> /200	1
3	Azide	<i>M</i> /500	0
3	Azide	<i>M</i> /1,000	0
16	Arsenite	<i>M</i> /50	100
16	Arsenite	<i>M</i> /100	100
16	Arsenite	<i>M</i> /200	93
16	Arsenite	<i>M</i> /500	86
16	Arsenite	<i>M</i> /1,000	86
19	Cyanide	<i>M</i> /50	58
4	Cyanide	<i>M</i> /100	35
19	Cyanide	<i>M</i> /100	33
19	Cyanide	<i>M</i> /200	20
4	Cyanide	<i>M</i> /1,000	20
4	Cyanide	<i>M</i> /5,000	11
14	Carbon monoxide	100%	2
13	Carbon monoxide	100%	0
13	Carbon monoxide	10%	0
8	$\alpha,\alpha'$ -dipyridyl	<i>M</i> /100	94
8	$\alpha,\alpha'$ -dipyridyl	<i>M</i> /200	89
8	$\alpha,\alpha'$ -dipyridyl	<i>M</i> /500	90
6	$\alpha,\alpha'$ -dipyridyl	<i>M</i> /1,000	74
6	$\alpha,\alpha'$ -dipyridyl	<i>M</i> /2,000	5
6	$\alpha,\alpha'$ -dipyridyl	<i>M</i> /10,000	5

$$^1\% \text{ inhibition} = \frac{Q_{CO_2} (\text{normal}) - Q_{CO_2} (\text{poisoned})}{Q_{CO_2} (\text{normal})} \times 100$$

The carbon monoxide used in these experiments was prepared in a formic-acid-sulphuric acid gas generator and was washed through alkaline pyrogallol. Three to four liters of pure carbon monoxide or carbon monoxide mixed with varying amounts of pre-purified nitrogen were passed through each Warburg vessel. The vessels were then shaken for 10 minutes to equilibrate with the gas before tipping in the substrate.

Several concentrations of  $\alpha,\alpha'$ -dipyridyl were tried. Inhibition with this compound varied from 94% in *M*/100 concentration to 5% in concentrations of *M*/2,000 and lower.

The fermentation of 3-carbon compounds, as well as that of glucose, was inhibited by  $\alpha,\alpha'$ -dipyridyl (Table VI). This inhibition varied from 90% with pyruvate to 58% with *dl*-threonine. Pyruvate, in contrast to glucose, was fermented by washed cells from a low-iron medium. This fermentation was likewise inhibited by  $\alpha,\alpha'$ -dipyridyl, the per cent inhibition being essentially the same as that obtained with cells from a high-iron medium. It is of interest to note that *M/500*  $\alpha,\alpha'$ -dipyridyl caused 90% inhibition of both glucose and pyruvate fermentation.

TABLE VI

*The Effect of M/500  $\alpha,\alpha'$ -dipyridyl on the Fermentation of 3-carbon Compounds by Cl. tetani*

Expt. No.	Substrate	Inhibition per cent
9	<i>dl</i> -serine	77
10	<i>dl</i> -threonine	58
10	Pyruvate	90
14	Pyruvate	90
20 <sup>1,2</sup>	Pyruvate	85
21 <sup>1</sup>	Pyruvate	83

<sup>1</sup> *M/200*  $\alpha,\alpha'$ -dipyridyl used.

<sup>2</sup> Organisms grown in low iron.

## DISCUSSION

The analysis of the breakdown products of glucose formed by *Cl. tetani* showed that the principal products of fermentation were ethyl alcohol, butyl alcohol, carbon dioxide, and hydrogen. Very small amounts of volatile acid and lactic acid were found. Although experiments on a micro scale indicated that the fermentation was essentially of an alcoholic nature, in which two moles of  $\text{CO}_2$  and two moles of alcohol were formed for each mole of glucose fermented, larger scale experiments gave lower yields of these products. The recovery accounted for approximately 75% of the carbon consumed. These results are comparable to those obtained by Friedemann and Kmiecik (1941) and Slade *et al.* (1942) for the glucose fermentation of *Cl. welchii*, with the exception that lactic acid was not present here in significant amounts. Our data indicated that only traces of lactic acid were formed during the fermentation of glucose by *Cl. tetani*, whether the organisms were grown in high or low iron concentrations.

The role of iron in the fermentation of glucose by the "TA" strain of *Cl. tetani* was investigated. It was found that the breakdown of glucose by washed suspensions of the organisms was far more complete when the organisms were grown in a medium containing an excess of iron than when they were grown in an iron-deficient medium. Pappenheimer and Shaskan (1944) showed that the fermentation of glucose by *Cl. welchii* was most efficient when the growth medium contained a carefully controlled amount of iron, and that when these bacteria were grown in a medium which was deficient in iron the fermentation was far less efficient and only minimal quantities of gas and volatile acids were produced. The activity of washed suspensions of *Cl. tetani* was progressively greater as the organisms were grown in media which contained increasing amounts of iron. A direct relationship of the  $Q_{CO_2}$  to the iron concentration was demonstrated.

Preliminary experiments indicated that inorganic iron added to washed suspensions had no effect on their activity. Similarly, Pappenheimer and Shaskan (1944) found that when iron was added to washed suspensions of *Cl. welchii* which were deficient in iron, there was no immediate acceleration of  $CO_2$  and  $H_2$  production, but that a small one was observed as fermentation proceeded. It was felt that the iron which caused increased activity of the organisms when added to the growth medium might be contained in an enzyme or coenzyme. Heat-treated suspensions of organisms grown in a medium containing an excess of iron caused a stimulation of glucose fermentation by unheated washed suspensions. Heat-treated suspensions of organisms from a medium deficient in iron also caused a stimulation of glucose fermentation (although somewhat less) by unheated washed suspensions. The results indicated that an iron-containing enzyme or coenzyme was elaborated by the organisms from both the high and low iron-content media during growth. It is possible that small amounts of an iron-containing enzyme were elaborated from minute quantities of iron present in the iron-deficient growth medium, which actually contained up to 0.02  $\gamma$  of iron per ml.

The possibility was considered that iron may have been necessary for the synthesis of this carbohydrate-splitting enzyme during growth without that metal entering into the structure of the enzyme itself, as has been suggested by Waring and Werkman (1944). However, the presence of iron in the washed cells was shown by the addition of  $\alpha,\alpha'$ -dipyridyl to the cells. A pink color resulted, even with those cells

grown in iron-deficient medium. The color was more intense with washed cells which had been grown in higher concentrations of iron.

Almost complete inhibition of gas production from glucose by washed cell suspensions was obtained when  $0.002\text{ M}$   $\alpha,\alpha'$ -dipyridyl was added. According to Pappenheimer and Shaskan (1944), this is evidence that iron is bound loosely in a postulated enzyme. This also precludes the existence of hemin or another iron-porphyrin compound as the iron-containing enzyme, for  $\alpha,\alpha'$ -dipyridyl does not combine with these (Sherman *et al.*, 1934). Pappenheimer and Shaskan suggest that the iron-containing enzyme involved in the glucose fermentation of *Cl. welchii* exerts its effect after an intermediate compound has been formed from glucose, and that this intermediate may well be pyruvic acid. We have seen that pyruvate is fermented by washed suspensions of the "TA" strain of *Cl. tetani*, and that this fermentation is almost completely inhibited by  $0.02\text{ M}$   $\alpha,\alpha'$ -dipyridyl.

Stephenson (1939) and others have shown that members of the genus *Clostridium* contain none of the cytochrome pigments. Spectroscopic examination of washed suspensions of cells of *Cl. tetani* from iron-rich and iron-deficient media, to which suspensions of sodium hydrosulphite had been added, revealed none of the characteristic cytochrome absorption bands. On the basis of these findings it would be expected that the fermentation was insensitive to cyanide. Although  $0.02\text{ M}$  potassium cyanide caused over 50% inhibition of fermentation, when this compound was tried in the usually employed concentration of  $0.001\text{ M}$  it caused only 20% inhibition. Thus the *Cl. tetani* fermentation, as well as the *Cl. welchii* fermentation, can be considered relatively insensitive to cyanide.

Azide, which is also believed to inactivate some iron derivative of hematin (Keilin, 1936), had no inhibitory effect on this fermentation.

Kubowitz (1934) claimed that all fermentations in which free hydrogen is concerned are inhibited by carbon monoxide. Although hydrogen was produced in the fermentation of glucose by *Cl. tetani*, carbon monoxide had no inhibitory effect. However, this is in accord with the finding that cyanide had no appreciable effect. It is not inconceivable that carbon monoxide and cyanide combine with only such iron compounds as hematin or its derivatives.

Arsenite caused considerable inhibition of fermentation, suggesting that the "TA" strain of *Cl. tetani* can effect the phosphorylation of glucose, since this compound competes with phosphate in the glucose

fermentation (Warburg and Christian, 1939). Arsenite may also inhibit the fermentation of glucose by blocking the breakdown of pyruvate (Krebs, 1933).

Finally, monoiodoacetate caused complete inhibition of fermentation, which gives a clue to one of the reactions involved in this scheme. According to Still (1940), this compound inhibits the action of triose-phosphate-dehydrogenase in the conversion of dihydroxyacetone-phosphate to 3-phosphoglyceric acid, occurring in the anaerobic breakdown of glucose.

### SUMMARY

Glucose is completely fermented by a mutant strain of *Cl. tetani*. The fermentation is of an alcoholic nature, with CO<sub>2</sub> and ethyl alcohol as the principal end-products.

The presence of iron in the growth medium is essential to this fermentation, and the glucose-fermenting activity of the organisms is directly related to the concentration of iron in the medium.

Inorganic iron does not stimulate glucose fermentation by washed suspensions of the organisms, but the addition of heat-treated suspensions does have a stimulatory effect.

The effect on this fermentation of various poisons, known to inhibit enzymes concerned in the catabolism of glucose, was investigated.

The presence of an iron-containing enzyme or coenzyme essential to this fermentation is postulated.

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# **Relative Value of Carotene in Vegetables for Growth of the White Rat**

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Received June 18, 1945

## **INTRODUCTION**

Recently it has been shown that the carotene in different foods and feeds is not as well utilized by the rat for storage of vitamin A in the liver as is carotene in cottonseed oil (6, 10, 12). Fraps and Meinke (10) found that the efficiency for different materials when compared with carotene in cottonseed oil ranged from 8% for turnip greens to 59% for alfalfa. Several workers have reported that the differences in the vitamin A activity between carotene in vegetables and carotene dissolved in oil are much less when the carotene is fed in small quantities for growth than when fed at higher levels for liver storage. Carotene in alfalfa products and in peanut hay was found to have practically the same vitamin A potency for rats as purified carotene dissolved in oil, while the carotene in yellow corn and in Johnson grass had slightly lower potencies (5). The potency of carotene in apricots was practically 100% of the expected value for rats (11). Likewise, there was close agreement between the amount of carotene in carrots and the bio-assay value as measured by growth and eye condition of rats (13).

According to Kemmerer and Fraps (7, 8) the carotene determined by the various methods used in the work referred to above did not consist entirely of  $\beta$ -carotene, but usually contained impurity A, neo- $\beta$ -carotenes U and B, and sometimes  $\alpha$ -carotene, in addition to the  $\beta$ -carotene. Neo- $\beta$ -carotene B (8) and  $\alpha$ -carotene (2) have 1/2 the vitamin A potency of  $\beta$ -carotene. Neo- $\beta$ -carotene U has 1/4 (9) to 1/3 (1) the vitamin A potency of  $\beta$ -carotene. Impurity A (14) has no vitamin A potency. The present study was designed to determine the vitamin A potency of the  $\beta$ -carotene equivalent of a number of plant materials

when fed to furnish relatively low levels of carotene. The  $\beta$ -carotene equivalent as used in this paper is the amount of  $\beta$ -carotene plus 1/4 the neo- $\beta$ -carotene U plus 1/2 the neo- $\beta$ -carotene B plus 1/2 the  $\alpha$ -carotene.

### EXPERIMENTAL

The relative vitamin A potencies of the  $\beta$ -carotene equivalents in the various foods and feeds were determined according to the U. S. P. X11 method with several modifications by Fraps, Kemmerer and Meinke (4). Briefly, this procedure consisted in comparing the growth of rats fed the test samples with the growth of rats fed a standard of purified carotene (90%  $\beta$ -carotene, 10%  $\alpha$ -carotene) dissolved in cottonseed oil. The rats were allowed free access to a basal diet composed of 59% corn starch (heated 24 hours at 170°C.), 22% heated casein, 9% non-irradiated yeast, 1% irradiated yeast, 4% salt mixture, 1% sodium chloride and 4% cottonseed oil. After the rats were depleted of vitamin A they were given the supplements twice weekly. The foods and feeds were fed at levels furnishing from 0.41 to 1.82  $\gamma$   $\beta$ -carotene equivalent daily and the standard at 0.6, 0.8 and 1.0  $\gamma$  daily. Ten rats were used for each level.

The carotenes in each sample and in the purified carotene solution were determined at the beginning of the feeding period. In order that the amount of  $\beta$ -carotene equivalent in each supplement should remain the same throughout the entire feeding period the amount of material necessary for each rat for each feeding period was weighed at the beginning of the feeding period, wrapped in wax paper, and kept frozen in the freezing unit of a refrigerator until fed.

Some work on dehydrated products done prior to the development of the methods for  $\beta$ -carotene equivalent is included in this paper. In this work, only the crude and pure carotene were determined (3); no attempt was made to estimate the  $\beta$ -carotene equivalents. These samples were kept in the refrigerator during the feeding period and the rats were fed as described above.

### RESULTS

Table I gives the results of tests on the 27 samples in which the  $\beta$ -carotene equivalents were determined. For purposes of comparison, the gain in weight of the rats receiving carotene dissolved in cottonseed oil was taken as 100. The average weight gained was calculated to a basis of a daily feeding of 1  $\gamma$  of carotene. These figures for the rats on the test samples were divided by the corresponding figure for the rats which received carotene in oil solution and the quotient was multiplied by 100. The carotene in all the samples tested except in the carrots was utilized practically as well for growth as was the carotene in oil. The average relative values except for carrots ranged from 94 for collards to 109 for alfalfa. The relative value for beet greens was 80, but since only one sample was analyzed it cannot be concluded that

TABLE I

*The Relative Efficiency of Carotene in Foods and Feeds to Carotene in Cottonseed Oil as Measured by Growth*

Name of food or feed	$\beta$ -carotene equivalent fed $\gamma/day$	Relative growth ( $\beta$ -carotene in oil as 100)
Spinach—(canned)	0.98	93.6
Spinach—(canned)	0.86	104.2
Spinach—(canned)	0.41	118.1
Sweet Potatoes (cooked)	0.80	106.1
Sweet Potatoes (cooked)	0.80	70.8
Sweet Potatoes (cooked)	0.62	116.1
Sweet Potatoes (cooked)	0.59	103.3
Swiss chard (cooked)	0.94	86.1
Mustard greens (cooked)	0.92	95.9
Mustard greens (cooked)	0.91	110.3
Alfalfa (low grade)	0.84	125.1
Alfalfa (high grade)	0.85	93.1
Turnip greens (cooked)	1.21	61.2
Turnip greens (canned)	0.90	123.3
Turnip greens (cooked)	0.85	105.5
Turnip greens (cooked)	0.56	90.0
Collards (cooked)	0.94	114.2
Collards (cooked)	0.82	73.7
Beet greens (cooked)	1.06	79.8
Apricots (canned)	0.67	103.4
Pumpkin (canned)	0.77	100.4
Carrots (cooked)	1.82	59.3
Carrots (cooked)	1.30	67.6
Carrots (cooked)	0.80	77.5
Carrots (cooked)	0.57	52.3
Carrots (raw)	1.62	50.2
Carrots (raw)	0.88	107.8

the carotene was less well utilized than it was in the other samples. The average relative value for 4 samples of cooked carrots was 64, which is considerably lower than the value obtained for the other materials.

TABLE II

*Relation of Carotene to USP Units of Vitamin A in Various Materials*

Name of food or feed	Pure carotene γ/g.	USP units per g.	USP units per γ carotene
Alfalfa	136.0	170	1.3
Alfalfa	310.0	236	.8
Alfalfa	140.0	208	1.5
Alfalfa	128.0	150	1.2
Alfalfa	276.0	390	1.4
Alfalfa	24.2	33	1.4
Alfalfa	24.2 (Crude)	33	1.4*
Beans, green string, powdered	6.7	9	1.3
Beans, dried string	12.9 (Crude)	21	1.6*
Beet tops, powdered	43.0	68	1.6
Lettuce, powdered	12.0	17	1.4
Mustard greens, powdered	188.0	293	1.6
Okra, powdered	4.0	4	1.0
Parsley, powdered	81.0	80	1.0
Peppers, dried green	21.3 (Crude)	36	1.7*
Peppers, powdered green	10.0 (Crude)	24	2.4*
Peppers, powdered green	6.9	8	1.2
Spinach, powdered	36.0	48	1.3
Turnip greens, powdered	166.0	194	1.2
Turnip leaves, powdered	27.0	42	1.6
Average (20)			1.4
(Standard deviation value of 0.8 and 2.4 excluded)			0.2

\* Calculated from crude carotene.

Table II contains the results of tests on the 20 samples in which the crude and pure carotene were determined but no attempt made to estimate the  $\beta$ -carotene equivalent. The value of 1  $\gamma$  of carotene in these materials ranged from 0.8 to 2.4 USP units. The average is 1.4 and is fairly close to the value of 1.67 which was given 1  $\gamma$  of  $\beta$ -carotene by the International Vitamin Conference in 1934. Taking into consideration that the inactive pigments were not eliminated by the analyses as they were in the work presented in Table I, the carotene in these materials was utilized on the average only to a slightly less degree, if any, than was the carotene in oil solution.

Mr. W. W. Meinke and Mrs. Lee De Mottier assisted materially in the analyses made for this work.

## SUMMARY

Except for carrots, the  $\beta$ -carotene equivalent of 27 samples of foods or feeds was as well utilized for the growth of rats as was the  $\beta$ -carotene equivalent of purified carotene dissolved in cottonseed oil.

In 20 tests on dried foods and feeds in which the amount of pure carotene was determined, but in which the amount of  $\beta$ -carotene equivalent was not determined, the value of 1  $\gamma$  of pure carotene ranged from 0.8 to 2.4 USP units. The average was 1.4 and this is close to the value of 1.67 given for 1  $\gamma$  of  $\beta$ -carotene by the International Vitamin Conference in 1934.

At a low level of feeding, sufficient for moderate growth, the vitamin A value of the  $\beta$ -carotene equivalent in plant materials appears to be equal to that of  $\beta$ -carotene dissolved in cottonseed oil. At high levels of feeding for storage of vitamin A in the liver, previous reports have shown that the  $\beta$ -carotene in plants is not nearly as well utilized as  $\beta$ -carotene in cottonseed oil.

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# The Effect of Fat and Sodium Pyruvate on Susceptibility of Thiamine Deficient Mice to Poliomyelitis Virus \*

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Received July 5, 1945

## INTRODUCTION

Thiamine-deficient mice show marked resistance to infection by poliomyelitis virus in contrast to the high susceptibility of mice fed a diet complete in all nutrients. Previous reports from both the Pennsylvania group (1, 2) and our laboratories (3) have established this fact.

In an early report Foster *et al.* (1) attributed this resistance to the decreased food intake in the deficient mice, but our data (3) indicated that simple caloric restriction was not the entire explanation. A later report by Foster *et al.* (2) gives data in substantial agreement with our findings. In contrast to thiamine-deficient mice, riboflavin-deficient mice show a slight resistance to infection with Lansing virus, but show no loss of appetite (4). Pantothenic-acid-deficient mice develop a definite increased resistance to Theiler's virus, but little or no change in susceptibility to Lansing virus (5).

This report describes experiments designed to obtain some insight into the mechanism of the resistance which develops in thiamine-deficient mice.

### *Statement of the Hypothesis*

Since it is well known that during thiamine deficiency certain metabolites, especially ketoacids, accumulate due to abnormal carbohydrate breakdown, it was important to determine whether these materials would interfere with the invasion of the virus. Two methods of approach were used. The first was designed to prevent the formation

\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. These studies were aided by a grant from the National Foundation for Infantile Paralysis, Inc.

of pyruvic acid by replacing the carbohydrate in the diet with fat, while still maintaining a thiamine-deficient condition in the mice.

It has been well established that rats and dogs require little thiamine when a considerable proportion of the carbohydrate in the ration is replaced by fat (6, 7, 8, 9). If thiamine *per se* is not essential for the metabolism of the virus, the virus should invade and thus cause paralysis as readily in mice placed on a high fat as when fed our regular diet. The second approach was to simulate the experimental deficiency conditions as closely as possible by adding pyruvate to the diet. While the blood pyruvate level is increased in thiamine deficiency (10, 11, 12) it is apparently difficult to increase this level by injection of the substance in normal chicks (13), in the dog (14), and in man (15). In contrast to this, Nitzescu and Angelescu (16) found that polyneuritic signs could be induced in pigeons by the injection of either lactate or pyruvate into the cranial cavity. It also seemed advisable to try the effect of pyruvate in conjunction with the high fat diets.

### EXPERIMENTAL

The diets used were similar to those previously described (1, 4, 5) and consisted of sucrose, purified casein, salt mixture, corn oil and adequate quantities of the crystalline vitamins. When sodium pyruvate was included, the substitution was made by removing an equal weight of the sucrose, salt and casein mixture. In the rations containing a low level of added fat, 20% of the calories, and in those containing a high level of added fat, 80% of the calories due to sucrose were replaced by an isocaloric equivalent of corn oil. In some of the high fat diets, the percentage of casein was increased to facilitate adequate incorporation of the corn oil in the diet. The exact composition of the various diets is given in Table I.

Mice from our colony were placed on the respective diets when 21 to 23 days old. The animals were injected intracerebrally either with Lansing strain poliomyelitis virus or with Theiler's GDVII encephalomyelitis virus after 9 to 15 days, depending upon when polyneuritic signs developed in the thiamine deficient control mice. Each litter of mice was divided among the various groups in the particular series, and each inoculated group was matched with an uninoculated control group, to obtain records of weight changes not related to the injection of the virus.

The animals tolerated 2, 3 and 4% of sodium pyruvate (17) with no untoward effects. If the mice ate an average of 2-3 g. of diet daily during the experimental period, the average consumption of pyruvate would be 60 to 90 mg. per day. The pyruvate was added to the diet adequate in thiamine, since we felt that this would be a severer test of the "toxic or blocking effect" on the virus.

*Series 32 (Lansing virus).* In the regular thiamine-deficient group (ration 103), none of the animals showed paralysis when the experiment was discontinued at 36 days. In the group with added thiamine, 36%

TABLE I  
*Percentage Composition of Thiamine-Deficient Diets*

	103 Regular basal	105a Low level of added fat	105b High level of added fat	106b Same as 105b plus increased level of vitamins	107b High fat basal plus thiamine- free liver
Sucrose	73	56 (53)	20.5 (15)	20.5 (15)	20.5 (15)
Purified casein	18	30 (28)	38.5 (28)	38.5 (28)	38.5 (28)
Salt mixture	4	4.25 (4)	5.5 (4)	5.5 (4)	5.5 (4)
Corn oil	5	9.6 (9.0)	35.0 (25.7)	35.0 (25.7)	35.0 (25.7)
Riboflavin, mg.	0.3	0.3	0.3	0.5	0.5
Pyridoxine, mg.	0.3	0.3	0.3	0.5	0.5
Nicotinic acid, mg.	0.5	0.5	0.5	1.0	1.0
Pantothenic acid, mg. (Ca salt)	2.0	2.0	2.0	4.0	4.0
Choline, mg.	300	300	300	600	600
Inositol, mg.	100	100	100	200	200
Para-aminobenzoic acid, mg.	100	100	100	200	200
Biotin, mg.	.005	.005	.005	1.0	1.0
Sulfited liver extract					2.0

Figures in parentheses are amount in grams of the constituent used in the high fat diets in which corn oil iso-calorically replaced the sucrose.

of the mice showed paralysis by the 15th day after inoculation. Only one mouse showed paralysis in the group receiving the basal with the low level of added fat, but this result was not surprising, as polyneuritic signs appeared in all the animals. When thiamine was added to this ration 62% of the mice were paralyzed in 13 days and 77% in 36 days. Thus, the addition of this level of fat did not alter the results either in the absence or presence of thiamine. In the group fed the high fat basal, 38% of the mice showed paralysis on the 13th day, while in the group with added thiamine there was an incidence of 50% at the same period. No further paralysis was seen in these groups when the experiment was terminated on the 36th day. The increased incidence of paralysis in the high fat group without thiamine supports our original hypothesis, but the number of animals in this series was so small that no definite conclusions were possible.

TABLE II  
*Per Cent of Mice Paralyzed after Inoculation with Lansing Virus*

Diet	No. of mice	Days after inoculation					Termination
		7th	9th	11th	13th	15th	
		Series 32					(36th day)
103 (Basal)	0	0	0	0	0	0	0
103 + 200 γ B <sub>1</sub>	14	14	21	29	29	36	50
105 a (Low fat)	14	7	7	7	7	7	7
105a + 200 γ B <sub>1</sub>	13	15	46	62	62	77	77
105b (High fat)	13	0	15	23	38	38	38
105b + 200 γ B <sub>1</sub>	14	14	21	43	50	50	50
103 + 200 γ B <sub>1</sub> + 3% NaPyruvate	13	15	15	15	15	15	31
		Series 33					(20th day)
103 (Basal)	28	7	7	7	7	7	7
103 + 200 γ B <sub>1</sub>	35	31	40	54	54	60	66
103 + 200 γ B <sub>1</sub> + 4% NaPyruvate	35	40	46	57	60	66	71
106b (High fat, high vitamin)	35	6	11	11	11	11	11
106b + 200 γ B <sub>1</sub>	35	40	46	60	71	71	71
		Series 39					(26th day)
103 (Basal)	28	0	0	0	0	0	0
103 + 200 γ B <sub>1</sub>	35	11	20	20	23	25	37
103 + 200 γ B <sub>1</sub> + 4% NaPyruvate	33	3	23	23	26	32	32
106b (High fat, high vitamin)	28	0	0	0	0	0	4
106b + 200 γ B <sub>1</sub>	34	3	6	12	18	24	44
106b + 200 γ B <sub>1</sub> + 4% NaPyruvate	33	12	15	15	15	24	27

The addition of 3% sodium pyruvate to ration 103 plus thiamine seemed to delay the incidence of paralysis, since on the 15th day only 15% of the mice showed paralysis as compared with 36% in the group without pyruvate. At the termination of the experiment 31% of the pyruvate group were paralyzed as compared with 50% in the group without the pyruvate.

*Series 33 (Lansing virus).* In this series ration 106b (increased vitamin content) was included because the consumption of the high fat diet was lower than that of ration 103. Seven per cent of the mice showed

paralysis in the ordinary thiamine-deficient group and 66% on the optimum thiamine diet. The addition of 4% of pyruvate to the optimum thiamine diet had no effect on the incidence of paralysis. Eleven per cent of the inoculated mice were paralyzed in the group receiving ration 106b as compared with 71% when the optimum level of thiamine was added to this ration. Thus the percentage of paralysis was very similar in the two deficient groups and in the two thiamine-supplemented groups regardless of the fat content of the diet. No thiamine-sparing action was found in the high fat group since definite polyneuritic signs were seen. When thiamine was added to this ration the percentage of paralysis was not only as high as usual but no significant differences were noted in the time of onset of the paralysis.

*Series 39 (Lansing virus).* The results in this series also indicate little thiamine-sparing action with the high fat diets. The addition of 4% sodium pyruvate to the regular thiamine-optimum diet produced a slight early resistance as compared with the control group without pyruvate, but at the end of the experiment, no significant differences

TABLE III  
*Per Cent of Mice Paralyzed after Inoculation with Theiler's Virus*

Diet	No. of mice	Days after inoculation					Termination
		7th	9th	11th	13th	15th	
		Series 43					(24th day)
103 (Basal)	32	0	3	16	19	19	19
103 + 200 γ B <sub>1</sub>	34	9	35	73	91	91	91
106b (High fat, high vitamin)	35	0	31	43	60	60	60
106b + 200 γ B <sub>1</sub>	35	3	31	75	97	100	100
107b (High fat + B <sub>1</sub> free liver)	35	9	17	43	51	51	51
		Series 47					(24th day)
103 (Basal)	35	0	0	0	0	0	0
103 + 200 γ B <sub>1</sub>	30	7	50	90	100	100	100
106b (High fat, high vitamin)	32	0	3	10	13	13	13
106b + 200 γ B <sub>1</sub>	30	3	33	67	77	77	80
103 + 200 γ B <sub>1</sub> + 4% NaPyruvate	32	0	31	60	88	91	94
106b + 200 γ B <sub>1</sub> + 4% NaPyruvate	15	0	20	53	53	60	60

existed. When 4 g. of pyruvate were added to the high fat diet plus thiamine no difference in the incidence of paralysis was noted from that in the group without pyruvate at the 13th day, but when the experiment was discontinued at 36 days, the percentage paralysis was 27% as compared with 44%. Pyruvate seems to be a little more effective on the high fat diets but the action is not nearly as complete as that produced by severe thiamine deficiency.

*Series 43 (Theiler's virus).* Both the deficient high fat group and the high fat plus thiamine group showed 31% mice paralyzed on the 9th day after inoculation, but by the 24th day only 60% of the animals in the high fat deficient group were paralyzed as compared with 100% in the high fat optimum group.

While mice grow normally on a highly purified diet complete in all the crystalline vitamins, we felt that more thiamine might be spared on a diet containing liver extract. A thiamine-free liver preparation equivalent to 2% sulfited liver extract was included in the high fat diet as a source of unknown accessory food factors. The addition of the sulfited liver to the high fat diet (107b) did not produce a better effect than that obtained in group 106b of this series. Fifty-one per cent of the mice were paralyzed as compared with 60% in the high fat deficient group, but when compared with the 19% in the regular thiamine-deficient group, the effect of high fat is apparent.

*Series 47 (Theiler's virus).* In this series, some slight sparing action of fat on thiamine was noted since the onset of polyneuritis in the high fat group occurred about 7 to 10 days later than in the regular low thiamine groups fed the 103 diet. Some change in the incidence of paralysis also was apparent since the high fat deficient group had 13% paralysis as compared with none in the deficient group on ration 103.

The findings in this series furnish the best evidence as to the action of pyruvate and/or high fat on susceptibility of mice to poliomyelitis. The addition of 4% sodium pyruvate to the complete diet produced an increased resistance during the first 12 days after inoculation as compared with the complete diet without pyruvate. At the 9th day the incidence in the two groups was 50 to 31% at the 10th day, 80 to 53%, at the 11th day, 90 to 60%. The difference in incidence decreased so that by the 24th day essentially 100% in both groups were paralyzed. The mice receiving the high fat diet seemed to have a greater sensitivity to the pyruvate than the group on ration 103. We have no explanation for this finding. Although few mice survived, a comparison of the high

fat diet plus thiamine and the same diet plus thiamine plus pyruvate shows some early protection in the group receiving pyruvate. When both groups receiving pyruvate are compared, differences between the high and low fat diets are small up to 12 days; but subsequently, the mice receiving the regular optimum diet plus pyruvate showed 94% as compared with 60% incidence in high fat diet plus pyruvate.

### DISCUSSION

It is difficult to induce a thiamine-sparing action by the use of increased dietary fat in mice under the conditions described in this report. Mice which receive enough corn oil to replace isocalorically 80% of the sucrose showed delayed signs of thiamine deficiency, but ultimately manifested the signs of the nutritional disease. The incidence of paralysis due to Theiler's encephalomyelitis virus was definitely increased in the groups receiving the 106b diet (high fat), yet the mice showed signs of polyneuritis. The explanation for these apparently diverse facts may be that the delayed onset of polyneuritis allowed the virus to establish itself and run its destructive course up to the time that the animal became thiamine-deficient.

The addition of pyruvate to the regular diet (103) had no effect on the growth or well-being of the mice. When the pyruvate was added to the regular diet plus thiamine, the pyruvate did not induce polyneuritis in mice. It may be that the amount of thiamine was in excess so that the effect of the pyruvate was nullified. The addition of pyruvate to the high fat diets also failed to produce polyneuritis. The mice were more sensitive to pyruvate on a high fat diet than on the regular diet, since many of these mice died earlier than usual, but no explanation for this finding is available at this time. Three of the four series which contained diets with added pyruvate showed a decrease in incidence of paralysis despite the lack of gross signs of deficiency. We must conclude that the addition of pyruvate to the diets decreased the paralytic incidence, but not so strikingly as in frankly thiamine-deficient mice.

The results with Lansing virus are more variable than those with Theiler's virus, but the tendency is the same. The resistance of thiamine-deficient mice to infection by poliomyelitis virus may be explained in part by the accumulation of pyruvate or other metabolites from incomplete carbohydrate breakdown. Other reasons for the increased resistance probably also exist, since the great difference in

incidence between deficient and optimally fed mice was not attained either by the use of pyruvate or of high fat.

We wish to thank Mr. Keith B. McCall for the preparation of the sodium pyruvate and Mrs. Edith S. Jones for her technical assistance throughout the course of these experiments.

### SUMMARY

The resistance of thiamine-deficient mice to infection with either Lansing poliomyelitis virus or Theiler's encephalomyelitis virus, can be explained only in part by the accumulation of pyruvate or of other metabolites from incomplete carbohydrate breakdown. The addition of 4% sodium pyruvate to a complete diet containing thiamine decreased the incidence of paralysis but not so strikingly as in frankly thiamine-deficient mice. The attempt to prevent the formation of pyruvic acid by using high fat diets was not wholly satisfactory because fat apparently has little sparing action on thiamine in the case of mice.

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# The Effects of Certain Salts on the Dissociation of Carboxyhemoglobin

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Received July 23, 1945

## INTRODUCTION

A considerable number of papers dealing with the effects of neutral salts on the dissociation of amino acids and proteins have been published.

Reviews are given by Batchelder (1) and by Edsall (2). In the case of certain amino acids, Batchelder and Schmidt (3) showed that the effects of salt mixtures are, in general, the sum of the effects of the individual salts and can be described on the basis of the Debye-Hückel theory. The effects of neutral salts on the dissociation of proteins are somewhat more complex due to the number of dissociable groups, the probability of overlapping constants, the shape of the protein molecule, etc. Although attempts have been made to describe the salt effects in specific terms (4-9), it has been necessary to make a number of assumptions. Cannan, Kibrick, and Palmer (5) have described their data by the simple equation,

$$\Delta pH_s = \alpha_s h + \beta_0, \quad (1)$$

where  $\alpha_s$  = slope of the curve,  $h$  = mean net charge per mole of protein,  $\Delta pH_s$  = difference in pH, and  $\beta_0$  = a constant factor for each curve so chosen as to make the curves intersect at pH 4.9, and indicating that the curves at different ionic strengths do not intersect at the same isoionic point ( $pI_s$ ). By choosing, in the case of egg albumin, the curve at a definite ionic strength of KCl as reference, it was found that  $\Delta pH_s$  is a linear function of  $h$  for the various curves studied. The same relation was found to hold for  $\beta$ -lactoglobulin, but the value for  $\beta_0$  was zero. On substituting salts of the  $MCl_2$  type for KCl at constant ionic strength, the dissociation curve was displaced toward a lower pH to an extent that increased with the ionic strength. The term  $\alpha_s$  was unaffected but  $\beta_0$  changed in sign. While the theoretical implications are not yet clear, it would seem that the effect of salts on the dissociation of the proteins studied are in line with the Debye-Hückel theory.

Cohn, Green, and Blanchard (10) found that the presence of NaCl has no effect on the maximum acid and base combining capacity of carboxyhemoglobin ( $HbCO$ ) but that it does displace the titration curve. The smallest effect on the pH is near the

isoelectric point ( $pI_a$ ) and is opposite in direction on either side of  $pI_a$ . Their data are in general agreement with those reported by others. This paper, so far as it appertains to the present investigation, will be discussed in greater detail later.

The object of the present work was to study the effects of inorganic salts and salt mixtures on the dissociation of HbCO. Comparatively little work has been carried out on this protein. Since hemoglobin differs from egg albumin and  $\beta$ -lactoglobulin in its amino acid content, isoelectric point, molecular weight, etc., it offered the opportunity of determining whether or not the generalizations that have been made on the basis of data obtained on the latter proteins, which are somewhat similar, also apply to HbCO.

#### PREPARATION OF MATERIALS

*Hemoglobin:* Horse red cells, kindly supplied by the Cutter Laboratories, were used as the source of this protein. The isolation procedure was a combination of those described by Altschul, Sidwell, and Hogness (11), Ferry and Green (12), and Welker and Williamson (13). The cells were washed three times with sodium chloride solution in the centrifuge, the temperature being maintained as close to 0° as possible. Sedimentation of the cells was expedited by adding 25 ml. of 0.1 M  $AlCl_3$  to each liter of the cell suspension. The cells were laked by adding 100 ml. of distilled water and 50 ml. of toluene to each 250 ml. of sedimented cells. After the laking was complete, the mixture was centrifuged. The upper layer was removed by suction. The laking process was repeated. Five tenths volume of aluminum hydroxide gel (14) was added to the hemoglobin solution with stirring and the mixture centrifuged. The procedure was repeated. The clear concentrated hemoglobin solution was placed in an ice-salt bath and the pH adjusted to 6.6 by careful addition of 0.1 N HCl with vigorous stirring. Sufficient cold ethyl alcohol was slowly added with vigorous stirring to give a concentration of 20–22% by volume. A small crop of crystals appeared. It was found necessary to keep the concentration and temperature of the alcohol as low as possible and to avoid any local concentration of alcohol. Crystallization of hemoglobin was completed by lowering the temperature of the mixture to –10° to –20°. The crystals were separated by centrifuging and washed 2 to 3 times with cold 25% alcohol. To the thick crystal paste, 0.5 volume of distilled water was added, followed by 1.0 N KOH with stirring until the crystals were dissolved. Any insoluble material was removed by centrifuging. The pH was about 7.8 to 8.0. The pH was now brought to 6.6 by addition of 0.1 N HCl, and the hemoglobin crystallized and washed with 25% alcohol as before. Dialysis was carried out in cellophane bags at about 1° against running distilled water by use of a rocking platform. The product was a thick crystal paste, bright red in color, and composed of prismatic rod-shaped crystals whose size depended on the speed of crystallization. It should again be emphasized that in all isolation steps the temperature should be kept low.

For purposes of preparing hemoglobin solutions, the molecular weight of this protein was taken as 67,000 and the nitrogen content as 16.86% (15) which gives a factor of 5.93 g. protein per g. of nitrogen. In agreement with Chibnall and coworkers (16), it

was found that Kjeldahl digestion of hemoglobin proceeds slowly and that periods of 8 and even more hours may be necessary to effect complete digestion, even when  $K_2SO_4$  was added to raise the boiling point and copper and selenium used as catalysts. In digesting for long periods of time, ammonia may be lost by volatilization (17-21), especially when the composition of the residue is such that it solidifies on cooling. Considerable excess of  $H_2SO_4$  should always be present during the digestion to avoid loss of ammonia. Eventually, the micro-Kjeldahl method in accordance with the procedure of Niederl and Niederl (22) was employed, 0.035 to 0.07 g. of hemoglobin being subjected to digestion.

The oxygen-combining capacity was used as a criterion for the purity of our hemoglobin preparations and especially as an indication of the extent to which conversion to methemoglobin or denatured hemoglobin had taken place as a result of manipulation and storage. The Van Slyke and Neil (23-27) manometric method was employed for this purpose. During the early part of the investigation, the iron content of hemoglobin was used as an additional criterion of purity. Both the method of Bernhart and Skeggs (28) and that of Saywell and Cunningham (29) as modified by Fortune and Mellon (30) were used. The former method requires larger amounts of hemoglobin than the latter procedure. The percentages of iron in our various hemoglobin preparations were: 0.342, 0.332, 0.342, 0.337, 0.336, 0.337 and 0.336. Zinnofsky (31) has reported 0.335% for the iron content of horse hemoglobin.

All inorganic salts used were of C. P. grade. In many instances they were further purified by recrystallization.

#### APPARATUS AND TECHNIQUE

The pH<sup>1</sup> changes during the titration of hemoglobin with HCl and with NaOH were determined by means of the glass electrode (National Technical Laboratory No. 015 below pH 9.0 and No. 1190 E above pH 9.0) using a saturated-KCl-calomel reference electrode and a saturated KCl bridge. The E.M.F. was measured with the aid of the Goyan (32) vacuum tube amplifier and a Leeds and Northrup student type potentiometer, the entire assembly being grounded and precautions taken to prevent current leakage. The glass electrodes were checked frequently. For estimating pH, the materials were placed in a cone-shaped vessel (32, 33). The temperature was maintained constant at 25°.

The titrations were carried out on HbCO instead of oxyhemoglobin as the former is less easily denatured by addition of acid or alkali. Before conversion to HbCO, each batch of stock solution of hemoglobin was subjected to the following analyses: oxygen-combining capacity, total nitrogen, residual dry weight, and in some cases, iron content. Only enough stock solution for several days was prepared, as hemoglobin is less stable in solution than in the frozen crystalline state. The acid and base combining capacity of oxyhemoglobin and carboxyhemoglobin are the same (34).

The titrations were limited to the range of pH 3.5 to 11.0 to avoid precipitation of hemoglobin chloride (10) which takes place at higher salt concentrations on the addition of acid, and to avoid various uncertainties at higher pH values. The con-

<sup>1</sup> Actually the paH is determined. When the term pH is used hereafter, the activity of  $H^+$  is implied.

centration of hemoglobin was kept constant in any series. The first titration was carried out on salt-free hemoglobin solutions; the second on a solution to which sufficient sodium chloride had been added to give an ionic strength of 0.012; the third on a solution having  $\mu_o = 0.10$ ; etc., up to  $\mu_o = 2.0$ . The concentrations, in terms of ionic strength, were on molarity basis ( $\mu_o$ ). All mixtures except the first in each series were at constant  $\mu_o$  (the amount of added salt was decreased with increasing amounts of acid or base added). The  $\mu_o$  of the first solution varied with the amount of acid or base added.

As there are no data at present on the activity coefficients of HCl and of NaOH in salt solutions containing hemoglobin or in hemoglobin solutions alone, it has been necessary to assume that the activity coefficients are the same in systems containing hemoglobin as in the corresponding systems containing only acid or base and salt. Although this assumption is not valid, it will probably not introduce any great error in the results. Values for  $\gamma_{HCl}$  in NaCl were taken from Harned and Mannweiler (35), those for  $\gamma_{HCl}$  in KCl from Harned and Harner (36) and Harned (37), and those for  $\gamma_{HCl}$  in LiCl from Harned and Copson (38). Values for  $\gamma_{HCl}$  in salt-free solutions were taken from Harned and Ehlers (39), Randall and Young (40), and MacInnes and Shedlovsky (41). For  $\gamma_{HCl}$  in CaCl<sub>2</sub>, see Harned and Brumbaugh (42). Since there are no available data for the activity coefficient of HCl in the various salt mixtures used in the present study, an average value was taken, assuming that the contribution of one salt to the activity coefficient is not affected by the presence of the other salt. Values for  $\gamma_{NaOH}$  in NaCl solutions were taken from Harned (43) and Harned and Cook (44) while those in salt-free solution were from Harned (45) and Harned and Hecker (46). Since no data are available for this quantity in KCl and LiCl, as well as in mixtures of these with NaCl, the activity coefficients of NaOH in these systems were assumed to be the same as in the corresponding NaCl solutions. The error involved would probably be very slight up to 0.4  $\mu_o$ .

Data for  $\gamma_{HCl}$  in NaCl + CaCl<sub>2</sub> and for  $\gamma_{NaOH}$  in CaCl<sub>2</sub> and in NaCl + CaCl<sub>2</sub> solutions are not available. These values were therefore obtained by pH measurements on solutions containing acid or base plus the salt solutions in the concentrations used when hemoglobin was present. In these systems the [H<sup>+</sup>] and [OH<sup>-</sup>]<sup>2</sup> bound by the protein were calculated by use of the following equations (2). For the systems containing hemoglobin and HCl + salt

$$\log \frac{C_2}{C_1} = pH_1 - pH_2 \quad (2)$$

where C<sub>1</sub> and pH<sub>1</sub> represent [H<sup>+</sup>] and p[H<sup>+</sup>], respectively, in the salt + HCl system and C<sub>2</sub> and pH<sub>2</sub> represent the corresponding values in the presence of hemoglobin. The quantity C<sub>1</sub> - C<sub>2</sub>, when calculated, gives the moles of H<sup>+</sup> bound by the protein per liter of solution. Similarly, for the systems of hemoglobin + NaOH + salt,

$$\log \frac{C_2}{C_1} = pH_2 - pH_1. \quad (3)$$

These equations eliminate use of activity coefficients as well as the necessity for using K<sub>w</sub>.

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\* The brackets denote concentrations of the quantities enclosed while parentheses denote the activities.

In those cases where the activity coefficients were taken from the literature, the following relations were used to calculate the acid or base bound by the hemoglobin:

$$\log [H^+] = pH - p\gamma_{H^+} \quad (4)$$

and

$$\log [OH^-] = (pK_w - pH) - p\gamma_{OH^-} \quad (4a)$$

The moles of acid or base bound by the hemoglobin per liter of solution is given by  $C_1 - C_2$  where  $C_1 = [H^+]$  or  $[OH^-]$  in the absence of the protein and  $C_2$  the corresponding value in the presence of the protein. Then  $C_1 - C_2$  divided by g. of hemoglobin gives the value of  $h$  or moles of acid or base bound per g. of protein. The moles of acid or base bound per mole of hemoglobin is given by  $h \times 67,000$ .

## RESULTS

The experimental results are shown graphically in Figs. 1-5. Although determinations were carried out to  $\mu_e = 2.0$  the values are not

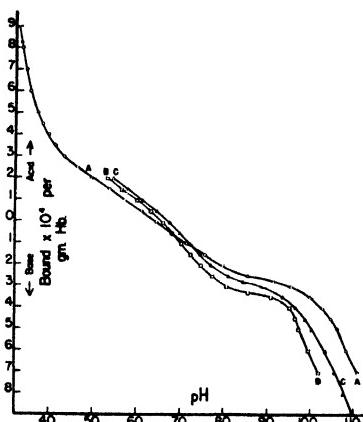


FIG. 1

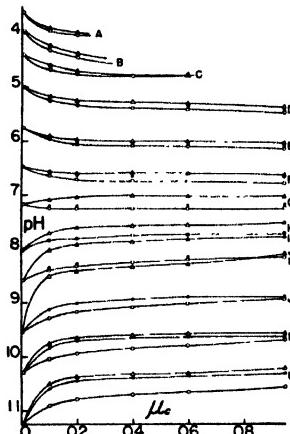


FIG. 2

FIG. 1. The Effect of Salts on the  $H^+$  Dissociation Curve of HbCO.

$\circ$ , In the absence of added salt (curve A);  $\square$ , In  $CaCl_2$  at  $\mu_e = 1.0$  (curve B);  $\Delta$ , In  $NaCl$  at  $\mu_e = 1.00$  (curve C). The points represent interpolated values taken from the smooth curves drawn through the experimental points.

FIG. 2. The Effects of  $NaCl$ ,  $CaCl_2$ , and a Mixture of  $NaCl + CaCl_2$  on the pH of HbCO Solutions at Constant  $h$ .

$\circ$ , In  $NaCl$ ;  $\Delta$ , In  $CaCl_2$ ;  $\bullet$ , In  $NaCl + CaCl_2$  ( $1 : 1$  on  $\mu_e$  scale). For the various curves, the values of  $h \times 10^4$  are: (A) 5.5; (B) 4.0; (C) 3.0; (D) 2.0; (E) 1.0; (F) 0; (G) -1.0; (H) -2.0; (I) -2.5; (J) -3.0; (K) -4.0; (L) -7.0. The values for pH were taken from the respective experimental dissociation curves for various values of  $\mu_e$  and at constant  $h$ .

shown in Figs. 2-4 since the curves would extend beyond the limits of the graph paper. When further values for  $\mu_e$  are plotted, the curves are a continuation of those shown in the figures. The effects of the various salts used on the dissociation of HbCO are in qualitative agreement with those found in the case of other proteins and with amino acids (3,5,6,10). The addition of salt to HbCO at any pH on the acid side of the isoelectric point leads to an increase of pH, while at any pH on the basic side of this point the pH decreases. This corresponds to increases in

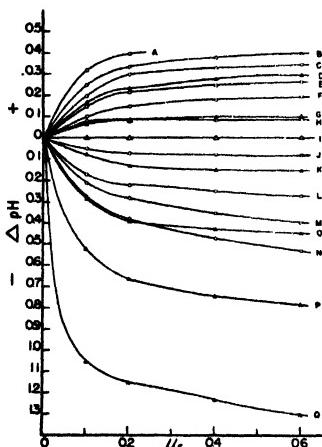


FIG. 3

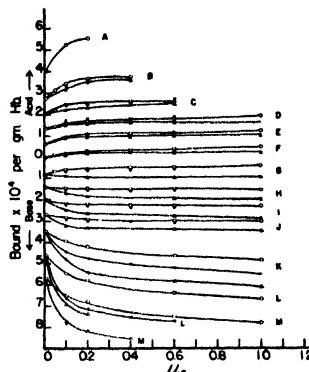


FIG. 4

FIG. 3. The Effect of Salts on the pH of HbCO Solutions at Various Values of  $h$ .  $\circ$ ,  $\text{NaCl}$ ;  $\Delta$ ,  $\text{CaCl}_2$ ;  $\Delta p\text{H}$  is the change in pH with increase in  $\mu_e$  at constant  $h$ , and was obtained from the experimental curves by taking the curve in the absence of salt as the standard of reference. Values of  $h \times 10^4$  are: (A)  $p\text{H} = 4.0$ ,  $h = 4.0$ ; (B)  $p\text{H} = 5.0$ ,  $h = 2.0$ ; (C)  $p\text{H} = 6.25$ ,  $h = 0.33$ ; (D)  $p\text{H} = 5.0$ ,  $h = 2.0$ ; (E)  $p\text{H} = 6.48$ ,  $h = 0$ ; (F)  $p\text{H} = 6.80$ ,  $h = -0.50$ ; (G)  $p\text{H} = 6.48$ ,  $h = 0$ ; (H)  $p\text{H} = 7.17$ ,  $h = -1.0$ ; (I)  $p\text{H} = 6.80$ ,  $h = -0.50$  ( $\text{CaCl}_2$ ) and  $p\text{H} = 7.37$ ,  $h = -1.25$  ( $\text{NaCl}$ ); (J)  $p\text{H} = 7.58$ ,  $h = -1.5$ ; (K)  $p\text{H} = 7.17$ ,  $h = -1.0$ ; (L)  $p\text{H} = 8.00$ ,  $h = -2.0$ ; (M)  $p\text{H} = 8.60$ ,  $h = -2.5$ ; (N)  $p\text{H} = 10.62$ ,  $h = -5.0$ ; (O)  $p\text{H} = 8.00$ ,  $h = -2.0$ ; (P)  $p\text{H} = 9.55$ ,  $h = -3.0$ ; (Q)  $p\text{H} = 10.62$ ,  $h = -5.0$ ; (R)  $p\text{H} = 9.55$ ,  $h = -3.0$ .

FIG. 4. The Effect of  $\text{NaCl}$ ,  $\text{CaCl}_2$ , and a Mixture of  $\text{NaCl} + \text{CaCl}_2$  on the Acid and Base Bound by HbCO

$\circ$ ,  $\text{NaCl}$ ;  $\Delta$ ,  $\text{CaCl}_2$ ;  $\bullet$ ,  $\text{NaCl} + \text{CaCl}_2$  (1 : 1 on the  $\mu_e$  scale). The values of  $h$  for each curve were obtained from the experimental dissociation curves at given values of  $p\text{H}$ . The  $p\text{H}$  values represented by the various curves are: (A) 4.00; (B) 4.50; (C) 5.00; (D) 5.50; (E) 6.00; (F) 6.50; (G) 7.00; (H) 7.50; (I) 8.00; (J) 9.00; (K) 10.00; (L) 10.50; (M) 10.75; (N) 11.00.

acid- and base-combining capacity, respectively. This effect ( $\Delta\text{pH}/\Delta\mu_e$ ) becomes greater the further the pH is removed from the isoelectric point, *i.e.*, the greater is the net charge on the protein molecule. The change ( $\Delta\text{pH}$ ) per unit increase in salt concentration ( $\Delta\mu_e$ ) decreases rapidly at higher salt concentrations, the greatest effect being between zero and 0.1  $\mu_e$ . Above 0.6  $\mu_e$ , there is very little increase in  $\Delta\text{pH}$  up to 2.0  $\mu_e$ .

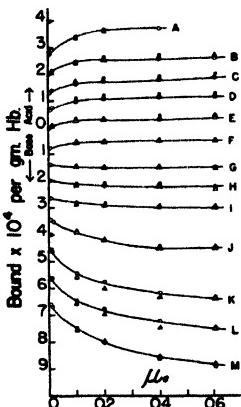


FIG. 5

A Comparison of the Effects of NaCl with those of Mixtures  
of NaCl + LiCl and NaCl + KCl

○, NaCl; Δ, NaCl + KCl (1 : 1); ●, NaCl + LiCl (1 : 1). Values for  $h$  were taken from the experimental dissociation curves at constant pH. The pH values represented by the various curves are: (A) 4.50; (B) 5.00; (C) 5.50; (D) 6.00; (E) 6.50; (F) 7.00; (G) 7.50; (H) 8.00; (I) 9.00; (K) 10.50; (L) 10.75; (M) 11.00.

The dissociation curve for HbCO in the absence of added salt is based upon observations on about 100 independent mixtures involving three separate preparations of HbCO (F, G, and H) and solutions varying in concentration of protein from 30 to 80 g. per liter. The data obtained when NaCl was present were obtained from all three of these preparations, while those for other salts and salt mixtures were obtained on single preparations of HbCO (G or H). All of these curves are based upon 30 or more independent pH determinations.

The data shown in Fig. 1 are in agreement with those reported by Cohn and coworkers (10) for Hb in low salt and 1.0  $M$  NaCl solutions and of Cannan and coworkers (5, 6) for egg albumin and  $\beta$ -lactoglobulin

and bear out the statement (5) that "the only observable effect of substituting KCl by  $MCl_2$  salts at constant ionic strength seems to be a parallel displacement of the whole dissociation curve toward a lower pH to an extent increasing with  $\mu$ ." This statement, however, does not completely describe the effects of divalent cations, since the displacement of the pH with increasing  $\mu_c$  is proportionally much greater at higher pH values. This effect was not obtained by Cannan and co-workers (5) since their study of  $MCl_2$  type salts covered only the pH 2.5 to 6.0 range. The main differences between the effects of the divalent and univalent salts appear above pH 7.0. The effects increase considerably above pH 7.0, become very large above pH 9.0, and reach a maximum at about pH 9.5. The differences in the effects of the two types of salts are not very large at reactions on the acid side of the isoelectric point.

Fig. 2 illustrates these effects in a different manner. The pH values at constant values of  $h^3$  were taken from the dissociation curves at various salt concentrations. These pH values were plotted against  $\mu_c$ . Fig. 4 is similar, except that the values of  $h$  were taken from the dissociation curves at given pH values and were plotted against  $\mu_c$ . In Fig. 3 the  $\Delta pH$  values were taken from the curves given in Fig. 2 and were plotted against  $\mu_c$ . These curves illustrate the magnitude of the pH changes with changes in  $\mu_c$  at constant values of  $h$ . They show the large effects of  $CaCl_2$  at higher pH values and that  $\Delta pH$  reaches a maximum at about pH 9.5. The effects of a mixture of NaCl and  $CaCl_2$  (1 : 1 on the  $\mu_c$  scale) shown in Fig. 4 are, in general, intermediate between those obtained when these salts alone were used; however, they follow those for NaCl alone more closely. Above pH 9.5, where the specific effect of  $CaCl_2$  is large, the difference between the curves for NaCl alone and those for NaCl +  $CaCl_2$  become much greater, and above pH 10.4 the effects of the salt mixture are almost identical with those of  $CaCl_2$  alone.

The effects of NaCl alone with those of NaCl + LiCl (1 : 1) and NaCl + KCl (1 : 1) are compared in Fig. 5. The curves for the mixtures are almost identical with those for NaCl. Several points obtained with a mixture of NaCl + LiCl + KCl, and several with LiCl and with KCl alone coincided almost exactly with those found for NaCl + LiCl, NaCl + KCl, and NaCl alone. Small differences may be due to liquid junction potentials which were not taken into consideration. There is

<sup>3</sup>  $h$  = moles of acid or base bound per g. of protein.

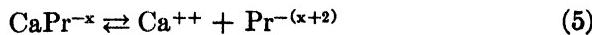
little or no specific ion effect in the univalent salts studied and no evidence of ion antagonism or anomalous behavior, such as Simms (47) reported, was obtained. The results are in agreement with the conclusions of Sörensen (48, 49) and Batchelder and Schmidt (3) that the effects of salt mixtures on the dissociation of proteins and amino acids are, in general, the sum of the effects of the individual salts.

The effect of salts on the so-called isoelectric point ( $pI_e$ ) or isoionic point ( $pI_i$ ) is of interest. In solutions of HbCO with no added acid or base, the pH increased with increase in salt concentration ( $\mu_c$ ). The pH of the salt-free solution was always close to 6.5. All of the pH values obtained were corrected to correspond to pH 6.48, which was taken as the average pH in the absence of salt, acid, or base. The increase in pH (or in  $pI_e$  if this is assumed to be the isoelectric point in the absence of salt) of the HbCO solutions in the absence of added acid or base was almost identical in all cases when univalent salts or salt mixtures were added. The maximum  $\Delta p\text{H}$  was 0.30 at 2.0 M salt concentration. Most of this increase, *i.e.*, 0.22 pH, occurred between 0.01 and 0.20  $\mu_c$ . The  $pI_e$  of HbO and HbCO is stated to be at pH 6.7 (34). This was obtained from measurements in buffer solutions. Even at pH 6.7 there is still a significant salt effect upon the addition of univalent salts.

The pH at which all of the curves intersect and  $\Delta p\text{H}/\Delta \mu_c$  is zero (a minimum) is at 7.37 (at  $h \times 10^4 = -1.22$ ). In the case of  $\text{CaCl}_2$ ,  $\Delta p\text{H}$  is much smaller, corresponding to a displacement of the curves in the presence of this salt to a more acid pH. This  $\Delta p\text{II}$  increases to a maximum of 0.15 pH at  $\mu_c = 0.6$ , then decreases somewhat at higher  $\mu_c$  values. The  $h - \text{pH}$  curves (dissociation curves) intersect at pH 6.8 (at  $h \times 10^4 = -0.45$ ). In the presence of the  $\text{NaCl} + \text{CaCl}_2$  mixture, the point of intersection is at pH 7.25 (at  $h \times 10^4 = -1.05$ ) and  $\Delta p\text{H}$  is intermediate between that when  $\text{NaCl}$  and  $\text{CaCl}_2$  respectively, are present alone.

## DISCUSSION

**1. Salt Effects and Complex Ions:** It has been shown repeatedly that polyvalent ions, including the alkaline earth cations, form complex ions with acidic compounds which have either two  $-\text{COOH}$ , one  $-\text{COOH}$  and an  $-\text{OH}$ , or two acidic  $-\text{OH}$  groups in close proximity, *i.e.* dicarboxylic acids, hydroxy carboxylic acids, phosphoric and boric acid, and proteins (50-55). Thus, in the case of calcium protein compounds,



Or, since a protein molecule can combine with a large number of calcium ions, and expressing the relationship by the mass law equation,

$$[\text{Ca}^{++}]^n \times [\text{Pr}^{-2n}] / [\text{Ca}_n\text{Pr}] = K_{\text{CaPr}} \quad (6)$$

The groups in the protein molecule responsible for the formation of complex ions when combined with a divalent cation to form a complex are stronger acids than the original dissociating groups. This probably explains the fact that calcium ions cause a shift of the dissociation curves to a lower pH than when NaCl at the same  $\mu_c$  is present (see Figs. 1-3). This is equivalent to decreasing the amount of acid bound to the protein at a given pH (see Figs. 1-4). This might also explain the fact that this effect increases with increasing pH (see Figs. 2 and 3). At a low pH, where the amount of free strong acid becomes greater in comparison to the amount bound, the relatively smaller acid strengthening effect of the calcium-protein complex would be largely masked. This effect becomes relatively more important with increasing pH.

The nitrogen of the imidazole group and the free amino groups would exist as the imidazolium and secondary ammonium ions, respectively, below about pH 5.5. Above this pH, the hydrogen will begin to dissociate from the imidazolium ion to give the imidazole group. The former has a residual charge of +0.77 while that of the latter is -0.33. Hence, as the imidazole groups are progressively formed with increasing pH, they will be able to take part in the formation of complexes, provided that the steric requirements are met, *i.e.*, sufficiently close proximity to a carboxyl group. This also holds true for the amino groups above about pH 9.5. The residual charge on the nitrogen of the substituted ammonium group is +0.89, while that on the amino group is -0.11. It would appear, then, that the increase of  $\Delta p\text{H}$  with increasing  $\mu_c$  might be explained qualitatively on this basis, *i.e.*, the greater possibility or capacity for complex formation with increasing pH (see Figs. 2 and 3).

Abels (56) has assumed that the carboxyl and hydroxyl groups (not necessarily phenolic hydroxyls) are involved in forming complexes with calcium since gelatin, which lacks tyrosine, combines with as much  $\text{Ca}^{++}$  as does egg albumin. Acetylated egg albumin, with the  $-\text{NH}_2$  and  $-\text{OH}$  groups blocked, combined with no  $\text{Ca}^{++}$ , while deaminized egg albumin combined with more of these ions than the untreated protein. The increase of  $\text{Ca}^{++}$  bound by deaminized egg albumin could easily be due to an increase in hydroxy groups since

$-OH$  has a -0.2 residual charge while  $-NH_2$  has a -0.11 charge. This would tend to increase the combination between protein and calcium.

This may not be the whole story. Other factors, perhaps, may also be involved. However, this tentative explanation fits the facts in a qualitative manner. Presumably the decrease in  $-\Delta pH$  or in  $h$  (Figs. 3 and 4) on the acid side of pH 6.5 might be partially or wholly due to the lower activity of  $Ca^{++}$  as compared with that of  $Na^+$ , the activity of calcium ions being further reduced by complex formation. However, on the basic side of neutrality, the change in pH (or  $h$ ) is opposite to that which would be predicted on this basis. The fact that the curves obtained with  $NaCl + CaCl_2$  approach those for  $CaCl_2$  at values above pH 9.0 and become almost identical above pH 10.4 (Figs. 2 and 4) seemingly indicates that the effect is due to complex formation and that the effect of  $Ca^{++}$  in the mixture is sufficiently great to overshadow the smaller effect due to  $Na^+$ . It would appear that two factors are involved: (a) a general ionic strength effect and (b) a specific effect of the divalent cation, the former increasing both the acid and base bound. Thus the net effect is a balance between, or an average of, these effects, which partially cancel out on the acid side and augment each other on the basic side of neutrality. Presumably, at lower pH values, complex formation plays the smaller role and the greater effect is due to ionic strength. Hence the effects of  $CaCl_2 + NaCl$  are intermediate between those for  $NaCl$  and those for  $CaCl_2$ . On the other hand, at higher pH values, the effects due to complex formation assume greater importance and determine the extent of the salt effects to a greater degree. Hence, the effect of  $CaCl_2 + NaCl$  approach those of  $CaCl_2$  in magnitude, since the effect of the  $Ca^{++}$  in the mixture predominates.

*II. Effects on the Isoelectric Point:* The present data indicate a definite salt effect on the isoelectric point of HbCO (see Figs. 1-3). Due to the method of preparation, the HbCO crystals, when dissolved in water, should yield a solution whose pH should have been close to the  $pI_e$  of this protein at zero ionic strength.

The  $pI_e$  of HbO and HbCO is usually stated to be at pH 6.7 (34). This value was obtained by measurements in buffer solutions. Since the  $pI_e$  is generally considered to be sensitive to changes in  $\mu$ , the above value would not represent the true  $pI_e$  at zero  $\mu$ . The true  $pI_e$  would be located at a lower pH. The pH obtained for the salt-free hemoglobin solutions may be lower than the true  $pI_e$  since no precautions were

taken to exclude  $\text{CO}_2$ . Crystallization of our hemoglobin took place at pH 6.6. This was lowered to 6.5 on dialysis and resolution in water (see Fig. 1, curve A). This pH change may also have been due to a more complete removal of salts during the dialysis. Whether the  $\text{pI}_e$  is taken to be at 6.5 or 6.7, it is still sensitive to changes in  $\mu$ , *i.e.*, the pH increases with increasing  $\mu$  (see Figs. 1 and 2). Cohn and coworkers (10) state that the effect of increasing the  $\text{NaCl}$  concentration is to increase the pH on the acid side of  $\text{pI}_e$  and to decrease it on the basic side, the effect being smallest near the  $\text{pI}_e$ . Their data do not indicate where this point is located. Their titration curves, in the absence of added salt and in 1.0 M  $\text{NaCl}$ , respectively, intersect at pH 6.5, which would indicate that this must be the isoionic point since the salt effect is minimum at this point. This is somewhat at variance with the present results. The curves obtained with various  $\text{NaCl}$  concentrations intersect at pH 7.37 (see Figs. 1 and 3). This was also found to be the case when mixtures of  $\text{NaCl} + \text{LiCl}$  and  $\text{NaCl} + \text{KCl}$  were used. This represents a value for  $h (\times 10^4)$  of  $-1.22$  on the basis of the system used, assuming a zero  $h$  value to be at the pH corresponding to zero added acid or base. Theoretically, the zero value for  $h$  should be at  $\text{pI}_i$ . If it is justifiable in identifying  $\text{pI}_i$  as that point on the dissociation curve which is independent of  $\mu$ , then, under these conditions, the  $\text{pI}_i$  should be located at pH 7.37.

The shift of the dissociation curves in the presence of  $\text{CaCl}_2$  at constant  $\mu_e$  to lower  $\text{pH}$  values as compared with corresponding values in the presence of univalent salts is in line with the effect on the isoelectric point as noted in the present investigation. The  $\Delta\text{pH}$  at  $h \times 10^4 = 0$  was only  $+0.15$  pH as compared to  $+0.30$  pH in the presence of  $\text{NaCl}$ . This would cause the family of curves to intersect at a lower pH value. In the presence of  $\text{CaCl}_2$ , the pH at the point of intersection was 6.8, which corresponds to  $h \times 10^4 = -0.45$ . This should correspond to  $\text{pI}_i$  as defined under these conditions. In the presence of  $\text{NaCl} + \text{CaCl}_2$  the point of intersection was at pH 7.25 ( $h \times 10^4 = -1.05$ ). These results point to the conclusion that the effects of salt mixtures on the dissociation of  $\text{HbCO}$  are, in general, the sum of the effects of the individual salts present in the mixture.

### III. Theoretical Treatment of the Data:

The theoretical curves calculated by Cannan and coworkers (5, 6) for egg albumin and  $\beta$ -lactoglobulin fit the experimental data for these two proteins remarkably well considering the assumptions that of necessity had to be made. However, in the case

of HbCO the curves described by these equations do not fit the data so well. Hemoglobin contains 174 reactive groups as compared to 92 for egg albumin and 104 for  $\beta$ -lactoglobulin. Of these 33 are imidazole groups as compared to 5 and 6 for egg albumin and  $\beta$ -lactoglobulin, respectively. Hence, the middle section of the hemoglobin dissociation curves is much steeper than is the case of the other two proteins. Hemoglobin contains 4 groups having  $pK'$  values around 4.8. These may represent carboxyl or some other acidic group connected with the porphyrin nucleus. Other proteins do not have comparable groups. As a consequence of these differences in composition and structure, it is to be expected that there will be differences in properties, especially in those relating to acid-base combination. A greater overlapping of  $pK'$  values is to be expected. This makes it difficult, in the case of hemoglobin, to assign  $pK'$  values, where overlapping occurs, to specific groups in the molecule. This was emphasized by Rykland and Schmidt (57) in the case of sulphydryl compounds.

An attempt was made to describe the data for HbCO by employing the various equations suggested by Cannan and coworkers (5-7) and which, in the case of egg albumin and  $\beta$ -lactoglobulin solutions at widely varying salt concentrations, were found to compare very favorably with the corresponding experimental curves. However, in the case of HbCO, the theoretical curves so calculated and using consistent values of  $w^4$  for the various salt concentrations, do not fit the curves obtained from experimental data, except in certain limited regions of the curves. Various probable values of  $w$  were tried with limited success.

It would appear, then, that these equations are not of general application in the case of proteins. The magnitude of  $n$  and  $m^5$  seems to be of importance. Thus for egg albumin and  $\beta$ -lactoglobulin, respectively,  $n$  is 41 and 46 and  $m$  is 51 and 58 while, in the case of hemoglobin, the value of both  $n$  and  $m$  is 87. The presence of the large number of imidazole groups in this protein is probably a factor involved. The recognized ellipsoidal nature of the hemoglobin molecule may also be a contributing factor, since the choice of the apparent radius will profoundly affect the magnitude of both  $b$  and  $w$ . In the equations mentioned above, the entire ionic strength effect resides in the term  $w$ . Hence the values assigned to this factor are of the utmost importance in describing changes in the dissociation curves of proteins resulting from changes in salt concentration.

In connection with the use of the following equation given by Cannan (7), *viz.*,

$$\text{pH} = \text{pH}_{\text{mid.}} - \log \frac{1 - \alpha}{\alpha} - 0.868 w m (\alpha - 0.5) \quad (7)$$

<sup>4</sup>  $w$  is a distance factor in the equations of Linderström-Lang.

<sup>5</sup>  $m$  = no. of groups of a given type, *i.e.*,  $-\text{COOH}$ , etc. and  $n$  = no. of groups having a positive charge.

it was found that good agreement could be obtained by multiplying the term  $[-\log(1 - \alpha/\alpha) - 0.868 w m (\alpha - 0.5)]$  by some purely empirical factor such as 1/3, 1/2, or 2/3. For the section of the curves representing the amino groups, good agreement with the corresponding section of the experimental curves was obtained by multiplying the above term by 1/3 for the curve in the absence of salt, by 1/2 for  $\mu_e = 0.1$ , and by 2/3 for  $\mu_e = 1.0$ . For the segments representing the imidazole groups, no simple relationship was found, better agreement being obtained by using the values as calculated from the above term without the use of any additional factors. In the case of the segments of the curves representing the carboxyl groups, the situation was even more complex. In most cases only a very small factor in addition to the  $\log(1 - \alpha/\alpha)$  term was necessary, indicating that, over most of the range of this segment, the dissociation of the carboxyl groups is quite similar to that of a mixture of univalent acids with no interaction except in the regions where the imidazole groups presumably begin to contribute to the  $pK'$ . It appears that the failure of the theoretical equations employed is probably not due to improper choice of values for  $w$  of else the discrepancy would be more uniform over the course of the curve.

Cannan (8) has suggested the following equation for calculating the theoretical dissociation curves of proteins:

$$pH = pK' + v \log(\alpha/1 - \alpha) \quad (8)$$

where  $v$  represents the slope of the straight line obtained when  $\log(\alpha/1 - \alpha)$  is plotted against pH and  $pK'$  is an apparent constant corresponding to the pH where  $(\alpha/1 - \alpha) = 1.0$ . Both  $v$  and  $K'$  vary with  $\mu_e$ . The application of this equation to the calculation of theoretical dissociation curves for HbCO in solutions of varying ionic strengths is shown by the data given in Tables I and II. Values of  $v$  were taken from Fig. 6,  $v$  being the slopes of the straight lines obtained by plotting experimental values of pH corresponding to given values of  $\alpha$ , against  $\log(\alpha/1 - \alpha)$ . For the various segments of the curves, values of  $h$  were based on the number of groups assigned to a certain segment in accordance with the  $pK'$  values given by Cohn and coworkers (10). These values of  $m$ , multiplied by  $1.5 \times 10^{-4}$  to convert them to moles per g. of HbCO, were in turn multiplied by  $\alpha$  to give the number of groups dissociating a proton at a given value of  $h$ . Values for  $h$  are then obtained by subtracting the number so obtained algebraically from the

total acid-base combining capacity. The theoretical curves, calculated in accordance with the above, were in very good agreement with the corresponding experimental curves between the limits of 0.2 and 0.8 for  $\alpha$ . This agreement is unexpected in view of the simplicity of the equation used. The form of this equation indicates that the calculated curves are necessarily symmetrical about their mid-points. It also shows that, as  $v$  approaches 1.0, the curve approaches that of a symmetrical polyvalent acid with an intrinsic dissociation constant  $K'$  and with no interaction or overlapping between groups. Equation (8) would then take the form

$$\text{pH} = \text{p}K' + \log (\alpha/1 - \alpha) \quad (9)$$

The dissociation of the  $-COOH$  groups in hemoglobin follows this pattern very closely at ionic strengths of 0.1 and above (see Table I), values for  $v$  being 0.975 and 0.99 in 0.1  $N$  and 0.2  $N$  NaCl, respectively, as compared to 0.77 in salt-free solutions. The dissociation of the

TABLE I

*Calculated<sup>1</sup> and Observed Dissociation Data of HbCO in NaCl Solutions*

$\mu_e$			Salt free	0.10	0.20	0.60	1.00	Salt free	0.10	0.20	0.60	1.00
$\alpha$	$\log \frac{\alpha}{1-\alpha}$	$h \text{ moles/g} \times 10^4$	Obs. pH**					Calc. pH				
Carboxyl <sup>1</sup>												
0.1	-0.955	13.50	3.3					3.38	3.58*			
0.2	-0.602	12.20						3.51	3.75			
0.4	-0.175	9.58						3.65	3.93			
0.5	0	8.27	3.38	3.58	(3.62)†			3.82	4.16			
0.6	0.175	6.07	3.50	3.75				4.08	4.50			
0.7	0.369	5.65	3.63	3.92	3.98	5.55						
0.8	0.602	4.36	3.88	4.19	4.28							
0.9	0.955	3.06	4.30	4.64	4.72							
$v^†$			0.77	0.98	0.99							
Imidazole <sup>3</sup>												
0.1	-0.955	1.25	5.57	5.75	5.88	5.92	5.97	5.23	5.53			5.91
0.2	-0.602	0.76	5.92	6.13	6.21	6.25	6.32	5.87	6.10			6.37
0.4	-0.175	-0.23	6.63	6.77	6.82	6.85	6.88	6.67	6.78			6.92
0.5	0	-0.725	6.97	7.06	7.17	7.17	7.15	6.97	7.06			7.15*
0.6	0.175	-1.22	7.37	7.37	7.37	7.37	7.37	7.33	7.34			7.38
0.7	0.369	-1.72	7.78	7.68	7.62	7.58	7.57	7.70	7.65			7.63
0.8	0.602	-2.21	8.23	8.05	8.00	7.92	7.85	8.13	8.02			7.93
0.9	0.955	-2.71	9.05	8.72	8.60	8.50	8.40	8.80	8.59			8.39
$v$			1.89	1.60			1.30					

TABLE I—Continued

$\mu_0$			Salt free	0.10	0.20	0.60	1.00	Salt free	0.10	0.20	0.60	1.00
$\alpha$	$\log \frac{\alpha}{1-\alpha}$	$h \text{ moles/g.} \times 10^4$	Obs. pH**				Calc. pH					
Amino <sup>4</sup>												
0.1	-0.955	-3.8	10.20	9.94	9.82	9.68	9.57	10.14	9.94	9.86	9.68	9.51
0.2	-0.602	-4.4	10.43	10.20	10.10	9.96	9.85	10.42	10.22	10.14	9.97	9.87
0.4	-0.175	-5.6	10.73	10.55	10.45	10.30	10.20	10.75	10.56	10.48	10.31	10.21
0.5	0	-6.2	10.88	10.70	10.68	10.45	10.36	10.88	10.70	10.62	10.45	10.35*
0.6	0.175	-6.8	11.05	10.85	10.77	10.60	10.53	11.01	10.84	10.76	10.59	10.49
0.7	0.369	-7.4	11.20	11.00	10.90	10.73	10.67	11.16	11.00	10.91	10.74	10.64
0.8	0.602	-8.0	11.38	11.18	11.02	10.85	10.80	11.34	11.18	11.10	10.83	10.83
0.9	0.955	-8.6	11.52	11.22	11.13	10.96	10.90	11.62	11.46	11.38	11.21	11.11
$v$			0.78	0.80	0.79	0.80	0.80					
Amino + Guanidino <sup>5</sup>												
0.1	-0.955	-4.01	10.30	10.03			9.70	10.25	9.99			9.66
0.2	-0.602	-4.82	10.57	10.32			9.98	10.59	10.34			10.02
0.4	-0.175	-6.44	10.96	10.74			10.44	11.01	10.76			10.45
0.5	0	-7.25	11.18	10.93			10.63	11.18	10.93			10.83*
0.6	0.175	-8.06	11.39	11.12			10.80	11.35	11.10			10.81
0.7	0.369	-8.87	11.55	11.27			10.96	11.54	11.29			11.00
0.8	0.602	-9.68	11.72	11.50			11.12	11.77	11.52			11.24
0.9	0.955	-10.49	11.91	(11.72)			11.27	12.11	11.97			11.60
$v$			0.98	0.99			1.01					

† Extrapolated values in parenthesis.

† v is the slope of the straight line obtained when  $\log (\alpha/1 - \alpha)$  is plotted against pH for given values of  $\alpha$ . Values for v were obtained from Fig. 6.  $\alpha$  represents the fraction of the total groups which have dissociated a proton.

\* The italicized pH values correspond to  $\alpha = 0.5$  and hence are identical with  $pK'$ .

\*\* The experimental values of pH are plotted vs.  $\log (\alpha/1 - \alpha)$  in Fig. 6.

<sup>1</sup> Equation (8) was employed in the calculation of the data.

<sup>2</sup>  $pK' = \text{pH}$  when  $h = +8.27$ . M = 87 as moles/mole, or 13.05 as moles/g. protein  $\times 10^4$ .

<sup>3</sup>  $pK' = \text{pH}$  when  $h = -0.725$ . M = 33 moles/mole, or 4.95 moles/g.  $\times 10^4$ .

<sup>4</sup>  $pK' = \text{pH}$  when  $h = -6.20$ . M = 40 moles/mole, or 6.0 moles/g.  $\times 10^4$ .

<sup>5</sup>  $pK' = \text{pH}$  when  $h = -7.25$ . M = 54 moles/mole, or 8.1 moles/g.  $\times 10^4$ .

carboxyl segment of HbCO could not be studied at higher salt concentrations due to precipitation of hemoglobin chloride. Apparently, however, the presence of the neutral salt tends to reduce the dissociation of the carboxyl groups to that of a mixture of univalent acids having the same apparent intrinsic dissociation constant.

Fig. 6 shows that the overlapping and interaction between groups in the segment of the HbCO curve representing the dissociation of the

TABLE II  
*Calculated<sup>1</sup> and Observed Dissociation Data of HbCO in CaCl<sub>2</sub> Solutions*

$\mu_0$			0.1	0.2	0.1	0.2		0.1	1.0	0.1	1.0
$\alpha$	$\log \frac{\alpha}{1-\alpha}$	$h \text{ moles/g.} \times 10^4$	Obs. pH**	Calc. pH		$h \text{ moles/g.} \times 10^4$	Obs. pH**	Calc. pH			
<b>Carboxyl<sup>2</sup></b>						<b>Imidazole<sup>3</sup></b>					
0.1	-0.955	13.50				1.25	5.73	5.83	5.62	5.75	
0.2	-0.602	12.20				0.76	6.11	6.18	6.10	6.18	
0.4	-0.175	9.58				-0.23	6.68	6.70	6.69	6.69	
0.5	0	8.27	(3.55)	(3.62)†	3.55	3.62	-0.73	6.93	6.90	6.93	6.90*
0.6	0.175	6.97				-1.22	7.22	7.12	7.17	7.11	
0.7	0.369	5.65	(3.90)	3.99	3.91	-1.72	7.52	7.36	7.44	7.34	
0.8	0.602	4.36	4.12	4.27	4.14	-2.21	7.83	7.60	7.76	7.62	
0.9	0.955	3.06	4.57	4.68	4.48	-2.71	8.17	7.85	8.24	8.05	
v†			0.98	0.98			1.38	1.20			
<b>Amino<sup>4</sup></b>											
0.1	-0.955	-3.8	9.70	9.65	9.54	9.41			9.38		9.24
0.2	-0.602	-4.4	9.89	9.80	9.82	9.69			9.63		9.52
0.4	-0.175	-5.6	10.17	10.03	10.16	10.03			9.85		9.86
0.5	0	-6.2	10.50	10.17	10.50	10.17			10.00		10.00
0.6	0.175	-6.8	10.45	10.33	10.44	10.31			10.14		10.14
0.7	0.369	-7.4	10.60	10.50	10.60	10.48			10.30		10.30
0.8	0.602	-8.0	10.75	10.68	11.06	10.65			10.48		
v†			0.80	0.80					0.80		

†, ‡, \*, \*\*. These values have the same meaning as in Table I.

<sup>1</sup> Calculated by equation (8).

<sup>2</sup>, <sup>3</sup>, and <sup>4</sup>. Values for M and pK' are the same as given in the footnotes to Table I for the corresponding groups.

in imidazole groups is so great that the curves obtained by plotting pH against  $\log(\alpha/1 - \alpha)$  deviate from a straight line at both ends. This is an indication of a greater discrepancy between the experimental and theoretical pH values in this segment of the curve as compared to the corresponding differences in the segments representing the dissociation of the carboxyl and amino groups. Fig. 6 also shows that the slopes, v, for the dissociation of the amino groups in the presence of NaCl are parallel and equal to 0.80. This also holds true for those in the presence of CaCl<sub>2</sub> ( $v = 0.80$ ) if the points corresponding to  $\alpha = 0.1$  and  $\alpha = 0.2$  are neglected. This corresponds to the results reported for egg albumin by Cannan and coworkers (5). Although the value for v is the same in the case of both types of salts, K' is lower when the divalent salt is used. In the case of the dissociation of the imidazole groups, this is partially

true except that the values of  $v$  and  $K'$  are both smaller when  $\text{CaCl}_2$  is present. In the case of the  $-\text{COOH}$  groups, there is very little difference in the values of  $v$  and of  $K'$  when either type of salt is present.

A better agreement between the experimental and calculated values for the imidazole groups is obtained if the 13 groups with a  $pK'$  around 5.7 and the 20 groups having a  $pK'$  around 7.5 are treated separately. However, in the case of the segment of the curve beyond  $h \times 10^{-4}$

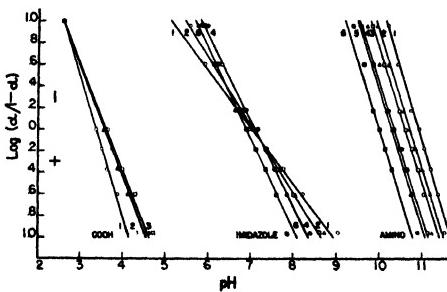


FIG. 6

The Salt Effect on the Slopes of the Curves Described by Equation (8)

- (1)  $\circ$ , Salt-free; (2)  $\Delta$ ,  $\mu_0 = 0.1$ ; (3)  $\square$ ,  $\mu_0 = 0.2$   $\text{NaCl}$ ; (4)  $\bullet$ ,  $\mu_0 = 1.0$   $\text{NaCl}$ ;
- (5)  $\blacktriangle$ ,  $\mu_0 = 0.1$   $\text{CaCl}_2$ ; (6)  $\blacksquare$ ,  $\mu_0 = 1.0$   $\text{CaCl}_2$ .

The slopes for the dissociation of the various groups are as follows:

Carboxyl	(1) $v = 0.78$ ; (2) $v = 0.975$ ; (3) $v = 0.99$ ; (5) $v = 0.97$ .
Imidazole	(1) $v = 1.89$ ; (2) $v = 1.60$ ; (4) $v = 1.30$ ; (6) $v = 1.20$ .
Amino	(1) $v = 0.80$ ; (2) $v = 0.79$ ; (3) $v = 0.80$ ; (4) $v = 0.80$ ; (5) $v = 0.80$ ; (6) $v = 0.80$ .

\* Amino + guanidino (1)  $v = 0.98$ ; (2)  $v = 0.99$ ; (4)  $v = 1.01$ .

\* Not shown in the figure.

= -3.2, a good agreement is also obtained if  $m$  is assumed to be 8.1 instead of 5.4 ( $\times 10^4$ ), i.e., the sum of both amino and guanidino groups. Table I shows that the value of  $v$  is very nearly 1.0 for all of these curves.

In view of the calculations made by Cohn and coworkers (10) on various portions of their HbCO dissociation curve, similar calculations were carried out using the present data. The following equation was used:

$$\frac{A^{-n}}{H_n A} = \frac{K'_1}{[H^+]} + \frac{2K'_1 K'_2}{[H^+]^2} + \dots + \frac{nK'_1 K'_2 \dots K'_n}{[H^+]^n} \quad (10)$$

This differs from their (10) equation only in that  $K_n' = K_n/\gamma_n$ . The term  $(\alpha/1 - \alpha)$  as used in equation (9) is equivalent to the term  $(A^{-n}/H_n A)$  in the above equation. This is merely the usual dissociation equation for acids and bases in which the term  $\frac{2K_1' K_2'}{[H^+]^2}$  is added to attempt to account for the effects due to overlapping of the constants. This is evident from the finding that, in the case of the  $-COOH$  groups at  $0.1 \mu_c$  and above, where  $v = 1.0$ , the second term of equation (10) drops out since it becomes too small to affect  $(\alpha/1 - \alpha)$ .

In order to apply equation (10) to the various segments of the HbCO dissociation curve, simultaneous equations were solved for  $K_1'$  at two different values of  $\alpha$ , *viz.*,  $\alpha_1$  and  $\alpha_2$ . In this way  $K_2'$  was eliminated, making possible the calculation of  $K_1'$ . The following expression was obtained:

$$K_1' = \frac{[H_1^+]^2 \left( \frac{\alpha_1}{1 - \alpha_1} \right) - [H_2^+]^2 \left( \frac{\alpha_2}{1 - \alpha_2} \right)}{[H_1^+] - [H_2^+]} \quad (11)$$

$K_1'$  was calculated for each group at each value of  $\mu_c$  using various values of  $\alpha_1$  and  $\alpha_2$  between 0.2 and 0.8. Values for  $pH_1$  and  $pH_2$  were taken from the experimental curves at values of  $h$  corresponding to  $\alpha_1$  and  $\alpha_2$ , respectively. The pH was converted to  $[H^+]$  by assuming that  $pH = \log \alpha_{H^+}$ . The 5 or 6 values of  $K_1'$  so calculated were then averaged to give the  $pK_1'$  value used for the given group and  $\mu_c$ .

Equation (10) was then solved for  $K_2'$  giving

$$K_2' = \frac{[H^+]^2 \left( \frac{\alpha}{1 - \alpha} \right) - [H^+] K_1'}{2K_1'} \quad (12)$$

This equation was solved at successive values of  $\alpha$  and the corresponding experimental values for  $[H^+]$ , and the various  $K_2'$  values, averaged as in the case of  $K_1'$ . Values for  $pK_1'$  and  $pK_2'$  are given in Tables III and IV. In comparing the data given in Table III with those in Table I, it is interesting to note that, in cases where the value for  $v$  in Table I is 1.0 or above,  $K_2'$  of Table III is zero or is a negative number.

Equation (10) was used in the form

$$\frac{\alpha}{1 - \alpha} = \frac{K_1'}{[H^+]} + \frac{2K_1' K_2'}{[H^+]^2} \quad (13)$$

for the calculation of the dissociation curves. Values of  $(\alpha/1 - \alpha)$  were calculated for given values of  $[H^+]$ . From these values,  $\alpha$  can easily be calculated. Then, taking  $\bar{h}$  as the maximum number of moles bound by hemoglobin,  $\alpha$  as the number of groups dissociating a proton,  $m_1$ ,  $m_2$  and  $m_3$  as the number of groups represented by the carboxyl, imidazole, and amino contributions to the curve, respectively, and  $h$  the acid or base bound at a given point on the curve (*i.e.*, the net charge), then

$$h_c = \bar{h} - \alpha m_1 \quad (m_1 = 87 \text{ carboxyl groups}) \quad (14)$$

$$h_i = \bar{h} - m_1 - \alpha m_2 \quad (m_2 = 33 \text{ imidazole groups}) \quad (15)$$

$$h_a = \bar{h} - (m_1 + m_2) - \alpha m_3 \quad (m_3 = 40 \text{ amino or}$$

$$54 \text{ amino and guanidino groups}) \quad (16)$$

TABLE III

*Calculated<sup>1</sup> Dissociation Constants for Various Groups in Carboxyhemoglobin*

$\mu_0$		Salt-free			0.1			1.0		
$\alpha_1$	$\alpha_2$	pH <sub>1</sub>	pH <sub>2</sub>	pK <sub>i'</sub>	pH <sub>1</sub>	pH <sub>2</sub>	pK <sub>i'</sub>	pH <sub>1</sub>	pH <sub>2</sub>	pK <sub>i'</sub>
Carboxyl <sup>2</sup>										
0.5	0.6	3.38	3.50	3.44	3.58	3.75	3.59			
0.5	0.7	3.38	3.66	3.42						
0.6	0.7	3.50	3.66	3.41	3.75	3.92	3.63			
0.6	0.8	3.50	3.90	3.35						
0.7	0.8				3.92	4.19	3.51			
0.7	0.9	3.66	4.30	3.38						
Av. pK <sub>i'</sub>				3.40			3.58			
Imidazole <sup>3</sup>										
0.1	0.2	5.57	5.92	6.53				6.32	6.88	6.88
0.2	0.4	5.92	6.63	6.47				6.88	7.15	6.97
0.4	0.5	6.63	6.98	6.70				7.15	7.37	7.09
0.5	0.6	6.98	7.37	6.88				7.37	7.57	7.19
0.6	0.7	7.37	7.78	7.10				7.57	7.86	6.94
0.7	0.8	7.78	8.23	7.31						
Av. pK <sub>i'</sub>				6.83						7.01

TABLE III—Continued

$\mu_0$		Salt-free			0.1			1.0		
$\alpha_1$	$\alpha_2$	pH <sub>1</sub>	pH <sub>2</sub>	pK <sub>1'</sub>	pH <sub>1</sub>	pH <sub>2</sub>	pK <sub>1'</sub>	pH <sub>1</sub>	pH <sub>2</sub>	pK <sub>1'</sub>
Amino										
0.2	0.4	10.43	10.73	11.21	10.20	10.55	10.88	9.85	10.20	10.53
0.4	0.5	10.43	10.88	10.97	10.55	10.70	10.79	10.20	10.35	10.45
0.5	0.6	10.88	11.05	10.90	10.70	10.85	10.76	10.35	10.53	10.34
0.6	0.8	11.05	11.38	10.98						
0.6	0.7	11.05	11.22	10.93	10.85	11.00	10.81	10.53	10.65	10.64*
0.7	0.8				11.00	11.18	10.77	10.65	10.80	10.61*
Av. pK <sub>1'</sub>				10.95			10.80			10.44
Amino + Guanidino <sup>1</sup>										
0.2	0.4	10.57	10.96	11.20				9.98	10.44	10.57
0.4	0.5	10.96	11.18	11.08				10.44	10.63	10.59
0.5	0.6	11.18	11.39	11.13				10.63	10.80	10.64
0.6	0.7	11.39	11.56	11.27				10.80	10.96	10.76
0.7	0.8	11.56	11.72	11.43*				10.96	11.12	10.81*
Av. pK <sub>1'</sub>				11.17						10.63*

\* Not included in average.

<sup>1</sup> Calculated with the aid of equation (11).

<sup>2</sup>  $m = 13.05 \times 10^{-4}$  moles/g. protein.

<sup>3</sup>  $m = 4.95 \times 10^{-4}$  moles/g. protein.

<sup>4</sup>  $m = 6.0 \times 10^{-4}$  moles/g. protein.

<sup>5</sup>  $m = 8.1 \times 10^{-4}$  moles/g. protein.

The values of  $h$  thus obtained can then be compared with similar values taken from the experimental curves at given values of  $\alpha$  (see Table V). In most cases the agreement is fairly good. In the case of the imidazole segment, however, the agreement is very poor.

An analysis of the data obtained on HbCO was carried out in a manner similar to that made by Cannan and coworkers (5) on egg albumin by employing the following equation (1). The values for  $\Delta pH$  were obtained by subtracting the pH values at the various values of  $\mu_0$  studied and at given values of  $h$  from the pH corresponding to the same value of  $h$  in the salt-free solutions. Thus, the curve representing the

dissociation of HbCO in salt-free solutions was taken as a standard of reference. The results are shown graphically in Figs. 7 and 8. Each line was obtained by plotting the  $\Delta\text{pH}$  values for the given  $\mu_e$  as a function

TABLE IV  
*Calculated<sup>1</sup> Dissociation Constants ( $K_2'$ ) for Various Groups in HbCO*

$\mu_e$	Salt-free		0.1		1.0		Salt-free		1.0	
$\alpha$	pH	pK <sub>2'</sub>	pH	pK <sub>2'</sub>	pH	pK <sub>2'</sub>	pH	pK <sub>2'</sub>	pH	pK <sub>2'</sub>
Carboxyl <sup>2</sup>										
pK <sub>1'</sub> ‡	3.40		3.58				6.83		7.01	
0.2							5.92	6.21	6.32	7.26
0.4							6.63	8.19	6.88	7.23
0.5	3.38	4.62	3.58	0			6.98	(?)	7.15	(?)
0.6	3.50	4.54	3.75	6.1			7.37	(?)	7.37	(?)
0.7	3.66	4.52	3.92	4.83			7.78	(?)	7.57	(?)
0.8	3.90	4.77*	4.19	4.31						
Av. pK <sub>2'</sub>	4.56		†					†		†
Amino <sup>4</sup>										
pK <sub>1'</sub> ‡	10.95		10.80		10.44		11.17		10.63	
0.2	10.43	(?)	10.20	(?)	9.85	11.72*	10.57	(?)	9.98	11.08
0.4	10.73	11.97	10.55	11.56	10.20	11.29	10.96	12.39	10.44	12.13
0.5	10.88	11.94	10.70	11.58	10.35	11.31	11.18	(?)	10.63	0
0.6	11.05	12.08	10.15	11.63	10.53	11.45	11.39	(?)	10.80	12.90
0.7	11.22	12.13	11.00	11.63	10.65	11.31	11.56	(?)	10.96	12.26
0.8	11.38	12.00	11.18	11.83	10.80	11.23	11.72	(?)	11.12	11.95
0.9	11.52	11.67*								
Av. pK <sub>2'</sub>	12.02		11.64		11.32			†		†

\* Not included in average.

† pK<sub>1'</sub> values obtained from Table III.

‡ Average has no significance.

(?) Negative values obtained.

<sup>1</sup> Calculated by means of equation (12).

<sup>2, 3, 4, 5</sup>. Values for m for respective groups are the same as given in Table III.

of h. For comparison, the corresponding data of Cannan and coworkers (5) for egg albumin in NaCl at 0.133  $\mu$  has also been plotted in Fig. 7.

Figs. 7 and 8 show that there is a straight-line relationship in the case of HbCO. However, the slopes of the lines are not the same over the

TABLE V  
Calculated<sup>1</sup> and Observed Dissociation Data for HbCO

$\mu_0$	Salt-free			1 0			Salt-free			1 0		
	pH calc.	h calc.	pH calc.	h calc.	pH calc.	h calc.	pH calc.	h calc.	pH calc.	h calc.	h obs.	
Carboxyl <sup>2</sup>												
	$pK_1' = 3.40$		$pK_1' = 3.58$									
	$pK_2' = 4.56$		$(+)$									
0.1												
0.2												
0.4												
0.5	3.38	8.56	3.58	8.27			8.27					
0.6	3.50	7.03	3.75	6.97			6.97					
0.7	3.63	5.96	3.92	5.80			5.65					
0.8	3.88	4.30	4.19	4.30			4.36					
0.9	4.30	2.75	4.64	2.66			3.06					
Imidazole <sup>3</sup>												
	$pK_1' = 6.83$		$(+)$									
	$pK_2' = 7.01$		$(+)$									
0.1												
0.2												
0.4												
0.5												
0.6												
0.7												
0.8												
0.9												
Amino <sup>4</sup>												
	$pK_1' = 10.95$		$pK_1' = 10.80$		$pK_1' = 10.44$							
	$pK_2' = 12.02$		$pK_2' = 11.64$		$pK_2' = 11.32$							
0.1	10.20	4.12	0.94	3.95	9.57	3.94	-3.8					
0.2	10.43	4.64	10.20	4.47	9.85	4.48	-4.4					
0.4	10.73	5.60	10.55	5.56	10.20	5.59	-5.6					
0.5	10.88	6.17	10.70	6.15	10.35	6.19	-6.2					
0.6	11.05	6.83	10.85	6.78	10.53	6.88	-6.8					
0.7	11.20	7.39	11.00	7.42	10.65	7.39	-7.4					
0.8	11.38	7.98	11.18	8.02	10.82	7.99	-8.0					
0.9	11.52	8.36	11.22	8.12	10.90	8.24	-8.6					
Amino + Guanidino <sup>5</sup>												
	$pK_1' = 11.17$		$pK_1' = 10.63$									
	$pK_2' = (?)$		$pK_2' = (+)$									
0.1	10.30	4.16	9.70	4.05	-4.01							
0.2	10.57	4.83	9.98	4.70	-4.82							
0.4	10.96	6.66	10.44	6.44	-6.44							
0.5	11.18	7.29	10.63	7.33	-7.25							
0.6	11.39	8.25	10.80	8.18	-8.06							
0.7	11.56	8.95	10.90	8.90	-8.87							
0.8	11.72	9.51	11.12	9.55	-9.68							
0.9	11.91	10.09	11.27	9.97	-10.49							

Values for  $pK_1'$  and  $pK_2'$  were taken from Tables III and IV respectively. Values of  $h \times 10^4$  were taken from the data plotted in Fig. 2 for the respective values of  $\alpha$  and  $\mu_0$ . (+) and (?) have the same meaning as given in Tables III and IV.

<sup>1</sup> The data were calculated with the aid of equation (13):

$$\frac{\alpha}{1 - \alpha} = \frac{K_1'}{[H^+]} + \frac{2K_1'K_2'}{[H^+]^2}$$

$\alpha$  was calculated for given values of  $(H^+)$ , using calculated values of  $K_1'$  and  $K_2'$  for the various groups. Using the known values of  $m$  for the various groups and the calculated values of  $\alpha$ , values for  $h$  can be readily obtained. The observed values of  $h \times 10^4$  were taken from the data plotted in Fig. 2, for the given values of  $pH$  and  $\alpha$ .

<sup>2</sup>, <sup>3</sup>, <sup>4</sup>, <sup>5</sup>.  $m$  has the same values for the various groups as those given in Table III.

whole range of the dissociation curve as is the case with egg albumin. Each figure, corresponding to a given  $\mu_o$ , consists of 4 or 5 straight lines, each having a different slope ( $\alpha_s$ ) and a different value of  $\beta_o$ , whereas, in the case of egg albumin, a single value of both  $\alpha_s$  and  $\beta_o$  sufficed to describe the whole curve. Segments I and IV, representing the extremi-

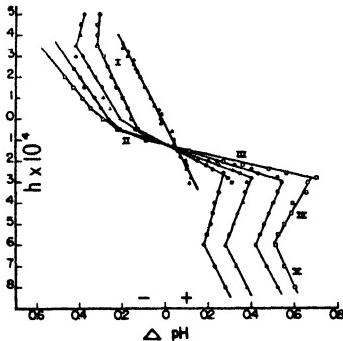


FIG. 7

The Effect of NaCl Concentration on the Relationship Between  $\Delta\text{pH}$  and  $h$   
 $\circ, \mu_o = 0.10; \Delta, \mu_o = 0.20; \bullet, \mu_o = 0.60; \square, \mu_o = 1.00; \blacktriangle, \mu_o = 0.133$ . Egg Albumin  
 (Cannan *et al.* (5)).  $\Delta\text{pH}$  is the difference in the pH at constant  $h$  between the curve taken as the standard of reference (*i.e.* the curve in the absence of salt) and the curve at the given value of  $\mu_o$ . The Roman numerals represent segments of the curve having different slopes.

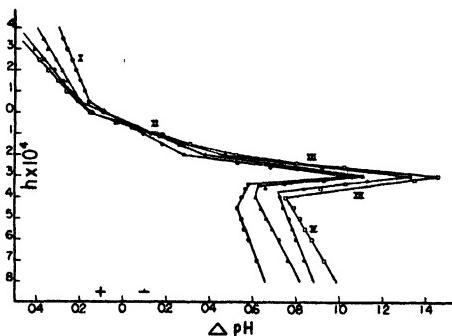


FIG. 8

The Effect of  $\text{CaCl}_2$  Concentration on the Relationship between  $\Delta\text{pH}$  and  $h$   
 $\circ, \mu_o = 0.10; \Delta, \mu_o = 0.20; \bullet, \mu_o = 0.60; \square, \mu_o = 1.00$ .  $\Delta\text{pH}$  has the same significance as noted in Fig. 7. The Roman numerals have the same meaning as in Fig. 7.

ties of the dissociation curves, both have a slope of the same order as the entire line obtained for egg albumin. However, segments II to IV have slopes of an entirely different order of magnitude. Segment IV has a negative slope. Fig. 8 indicates that this difference in the case of HbCO and egg albumin is even more marked when  $\text{CaCl}_2$  is present in the solution. The effects of  $\text{CaCl}_2$  on the pH of egg albumin solutions is to cause  $\beta_0$  to have negative values, while the corresponding effects on the pH of HbCO are to yield values of  $\beta_0$  that have the same sign as the representative values when  $\text{NaCl}$  is present.

If Cannan and coworkers (5) used the curve at  $0.067 \mu \text{ KCl}$  as the standard of reference for the  $\text{CaCl}_2$  curves, then the above difference is partially explainable on the basis of the observed differences in the effects of  $\text{NaCl}$  and  $\text{CaCl}_2$ , respectively, on the dissociation curves, *i.e.*, that the whole curve is displaced toward a lower pH when  $\text{CaCl}_2$  is present than is the curve at a similar value of  $\mu_e$  in  $\text{NaCl}$ . In that case, however, the numerical value of  $\beta_0$  in the case of  $\text{CaCl}_2$  would decrease rather than increase with increase in  $\mu_e$ . There is no evident explanation of the differences in the salt effects on the dissociation of the two proteins unless it is on the basis of the known differences in their chemical makeup. The larger number of imidazole groups in HbCO causes the dissociation curve to be much steeper in the neutral pH range than is the case of egg albumin. This fact could explain qualitatively the almost 5-fold increase in the  $\alpha_s$  values between  $h \times 10^4 = 0$  and  $h \times 10^4 = -3.0$  as compared to the values for egg albumin at the same  $\mu_e$  and within the same range of  $h$ . This increase in slope corresponds to proportional increases in  $\Delta\text{pH}$ . Since egg albumin has only 4 or 5 imidazole groups, as compared to 33 for HbCO, they would exert relatively little effect in this region of the curve. The imidazole group, when present in proteins, appears to be much more sensitive to changes in  $\mu$  than is the case with  $-\text{COOH}$  and  $-\text{NH}_2$  groups. The specific effects of  $\text{CaCl}_2$  attain a maximum in this range of  $h$  values. This may be in line with the fact that the imidazole residue (the N atom) possesses a residual charge of  $-0.33$  when in the uncharged form, *i.e.*, when the pH has increased to the point where  $\text{H}^+$  is dissociated leaving  $>\text{NH}$ . This would have a greater electrostatic attraction for  $\text{Ca}^{++}$  than either  $-\text{NH}_2$  or  $-\text{OH}$ .

Beyond  $h \times 10^4 = -6.0$  for the  $\text{NaCl}$ -containing solutions and  $h \times 10^4 = -4.0$  for those that contain  $\text{CaCl}_2$ , the slope ( $\alpha_s$ ) again becomes of the same order as it is above  $h \times 10^4 = 0$ . In the case of the

NaCl solutions, the slope of this segment remains constant with increasing  $\mu_e$  (compare with the constancy of  $v$  shown in Table I for the amino group dissociation at varying  $\mu_e$ ). In the case of the CaCl<sub>2</sub> solutions this is not strictly true.

In Figs. 7 and 8 the labeled segments correspond roughly to the following groups: I. carboxyl; II and III. imidazole; IV. amino; V. amino or guanidino. In some cases there is considerable overlapping, but the various segments having different slopes might reflect the difference in the salt effects on the various types of groups. It is to be expected that the salt effects would differ qualitatively and quantitatively as a result of differences in the groups themselves.

The complete data are on file in the University of California Library, Berkeley.

#### SUMMARY

1. A modified method for the preparation of horse HbCO is described.
2. The effects of various inorganic salts and salt mixtures on the dissociation of HbCO have been determined. These effects are shown to be qualitatively consistent with those obtained in the case of amino acids and proteins, *viz.*, an increase in pH on the acid side of the isoionic point and a decrease on the basic side with increasing salt concentration.
3. In general, the effects of salt mixtures are shown to be the sum of the effects of the individual salts. In cases where the effects are not strictly additive, complex formation is suggested as the contributing factor.
4. The effect of CaCl<sub>2</sub> is to shift the whole dissociation curve to a lower pH, the shift being relatively much greater at higher pH values. These specific effects are discussed on the basis of complex formation between Ca<sup>++</sup> and certain groups in the protein.
5. The isoelectric point is shown to be sensitive to changes in ionic strength.
6. An attempt was made to calculate theoretical dissociation curves employing 3 different equations. Theoretical curves were calculated at various salt concentrations and compared with the experimental data. In cases where the agreement was poor, the disagreement was assumed to be due to overlapping of and interaction between groups.
7. The apparent dissociation constants for the various groups involved in the dissociation of HbCO were calculated at several different values of ionic strength.

8. A comparison was made of the effects of salts on the dissociation of HbCO with those on the dissociation of other proteins. The differences were attributed to differences in composition and structure of the various proteins.

9. The salt effects are chiefly attributed to two factors. The first is an interionic attraction between the salt ions and the charged groups in the protein. This effect was assumed to be proportional to the ionic strength and independent of the type of salt. The second is a specific ion effect related to the ability of certain types of salts to form complexes with certain groups in proteins. This applies chiefly to polyvalent cations.

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## **Electrophoretic Investigation of Peanut Proteins**

### **II. Composition of Several Peanut Protein Fractions**

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Received July 26, 1945

#### **INTRODUCTION**

Together, arachin and conarachin, two protein fractions that were first isolated from peanut meal by Johns and Jones (1), account for nearly all of the protein of peanut meal, but the procedure for obtaining them is not conveniently applicable to the large scale operations which would be required if quantities of peanut protein were needed for incorporation in food products or for use in industrial products such as adhesives, sizes, paper coatings and fibers (2). In this connection it has been observed that high yields of protein (approximately 91% of the protein or 42% by weight of the meal) can be obtained very simply by adjusting a slightly alkaline extract of solvent-extracted peanut meal to approximately pH 4.5, the minimum solubility point of the proteins in the presence of all of the soluble substances of the meal (3). Inasmuch as the peanut is a rich source of high-quality food protein, a method by which high yields of such protein preparations can be obtained, as well as information concerning the number and relative abundance of the protein components present in the preparation, are important considerations if wide use of the isolated protein is contemplated.

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Furthermore, it has been shown (4) that protein preparations having certain unique physical properties can also be obtained quite simply and in good yields from peanut meal by successively adjusting a concentrated neutral or slightly alkaline extract of the meal first to pH 6.0 and then to pH 4.5. The protein obtained by precipitation at pH 6.0 forms as precipitated a plastic, fluid, stringy mass which can be pulled readily into fine threads and exhibits a marked gloss and sheen. The protein subsequently precipitated at pH 4.5 is granular and non-coherent, exhibiting no tendency toward stringiness or gloss. Because of these differing physical characteristics both of these preparations, as well as the preparation mentioned above, have possibilities for use in industrial products where the applicability of a protein preparation often depends upon whether or not it possesses certain desirable physical properties (2, 5). In the case of these two protein preparations, it is also of importance to ascertain in each the number and relative abundance of the protein components previously found to be present in the meal (6).

It is the purpose of this communication to present the results of an electrophoretic investigation of the protein preparations obtained by precipitation from peanut meal extracts at pH 4.5; of the small amount of the protein fraction which remains in the mother-liquor after the removal of the protein fraction precipitable at pH 4.5; and of the protein fractions obtained by precipitation from peanut meal extracts first at pH 6.0 and then at pH 4.5.

## EXPERIMENTAL

### *Electrophoretic Analysis of Peanut Protein Precipitated at pH 4.5*

One gram of peanut meal, prepared as described previously (6), was extracted at 1-3°C. with 5 successive portions of ammonia buffer (0.2 M ammonia and 0.1 M hydrochloric acid, pH 9.26 at 25°C., 0.1 ionic strength) and the extract (50 ml.), which contained 97.5% of the protein nitrogen of the meal, was brought to pH 4.5 by the addition of hydrochloric acid. The precipitate obtained contained 91.2% of the protein nitrogen of the meal but only a negligible trace of non-protein nitrogen as determined by its solubility in 1 M trichloroacetic acid at 25°C. The protein fraction was separated by centrifugation and was dissolved immediately, without washing, in ammonia buffer. The buffer solution of the protein was treated and analyzed electrophoretically in the Tiselius apparatus as previously described (6, 7).

A typical pattern for an ammonia buffer solution of the protein fraction precipitated at pH 4.5 is shown in Fig. 1, and the mobilities

of the several components are given in Table I. The three well-defined boundaries present in the descending pattern have mobilities of  $-5.9$ ,  $-5.1$  and  $-3.5 \times 10^{-5}$ , respectively. While these mobilities correspond fairly well with those for the components A, B and C which were previously shown to be present in the ammonia buffer extract of peanut meal, the major components show a tendency to the same type of pattern asymmetry reported for arachin (6). Similar results were obtained in other experiments on this fraction and on the fraction

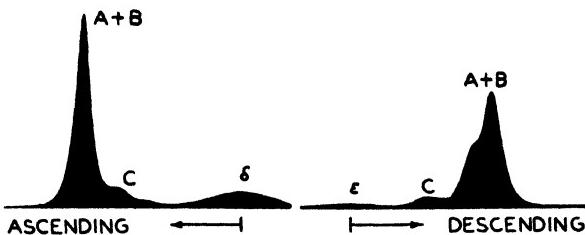


FIG. 1

Electrophoretic Pattern of Peanut Protein Precipitated at pH 4.5 After Electrophoresis for 16,680 Seconds in Ammonia Buffer at pH 9.26

precipitating at pH 6 (below). In some cases the major component boundary on the ascending pattern showed a distinct shoulder on its forward side as in the arachin pattern (6), or as in Fig. 3, and in some cases it was similar to Fig. 1, but in no case did it appear as on the pattern for the buffer extract of peanut meal (Fig. 1 of ref. (6)). In view of this, a pattern was obtained on an ammonia buffer extract which had been adjusted to pH 4.5 and was subsequently equilibrated with the ammonia buffer without removing any precipitate. This resembled Fig. 1 rather than the pattern of the untreated ammonia buffer extract indicating that the change in the major component boundary can be brought about merely by exposure to pH 4.5 and is probably not due to the removal of some substance reacting with the proteins when the precipitation at pH 4.5 is carried out.

The approximate distribution of the major and minor protein components and of the protein nitrogen of the meal in the fraction precipitated at pH 4.5 and in the various fractions to be described is given in Table II. To obtain the figures given in columns three and four of this Table, 10 g. portions of peanut meal were extracted and the protein fractions were precipitated exactly as described for the

TABLE I

*Mobilities and Estimates of the Relative Amounts of the Protein Components in Various Protein Fractions Obtained from Peanut Meal*

Ammonia buffer, 0.1 ionic strength; pH 9.26 at 25°C; field strength 3.1 volts per cm; protein concentration approximately 0.8%; mobility in  $\text{cm}^2 \text{ volt}^{-1} \text{ sec}^{-1} \times 10^6$  reduced to 0°C.

No.	Fraction Description	Protein Component					
		A		B		C	
		Mobility	per cent	Mobility	per cent	Mobility	per cent
1	Buffer extract of peanut meal <sup>1</sup>	-6.3	76	-5.1	11	-3.7	13
2	Protein precipitated at pH 4.5	-5.9	75	-5.1	17	-3.5	8
3	Protein remaining after removal of 2 <sup>2</sup>	—	—	—	—	-3.4 <sup>3</sup>	100 <sup>4</sup>
4	Protein precipitated at pH 6.0	-6.2	73	-5.3	19	-3.3	8
5	Protein precipitated at pH 4.5 after removal of 4 . . . . .	-6.2	34	-5.2	36	-3.3	30

<sup>1</sup> Taken from Table I of the preceding paper (6).

<sup>2</sup> Protein concentration approximately 0.2%.

<sup>3</sup> The figure given represents the average mobility of two components having mobilities of -4.2 and  $-2.6 \times 10^{-6}$ , respectively. The faster of these two components comprises approximately 52% of the total protein in this fraction.

<sup>4</sup> As indicated in the text, this fraction is probably not composed exclusively of the minor components but the amounts of A and B components present are too small to be detectable in the electrophoretic pattern.

1 g. portions used in preparing samples for electrophoretic analysis. These fractions were analyzed for nitrogen (corrected for ammonia) to determine the distribution of the original meal nitrogen. The figures given in the last two columns of Table II were obtained by multiplying the figures given for the percentage composition of each of the fractions (Table I) by the weight of the protein in each fraction. No attempt was made in Table II to indicate the distribution of the A and B components separately, but the two have been grouped as major components.

When the electrophoretic composition of the peanut protein fraction precipitated at pH 4.5 is compared with that of arachin and conarachin

TABLE II

*Approximate Distribution of the Protein and Protein Components in Various Fractions Obtained from Peanut Meal*

No.	Fraction Description	Protein Nitrogen <i>per cent of meal nitrogen</i>	Protein <sup>1</sup>	Components	
				Major	Minor
1	Buffer extract of meal <sup>2</sup>	97.5	4.53	3.94	0.59
2	Protein precipitated at pH 4.5	91.2	4.24	3.90	0.34
3	Protein from mother-liquor of 2	4.4	0.22	—	0.22 <sup>3</sup>
4	Protein precipitated at pH 6.0	75.6	3.52	3.24	0.28
5	Protein precipitated at pH 4.5 after removal of 4	15.0	0.70	0.49	0.21

<sup>1</sup> Protein = Protein nitrogen  $\times$  5.5; yields based on 10 g. meal used.

<sup>2</sup> Taken from Table II of the preceding paper (6).

<sup>3</sup> See footnote 4 of Table I.

(6), it is obvious that marked differences exist. Although the protein fraction precipitated at pH 4.5 is not truly representative of the total protein of the peanut since, as will be shown in the following section, an appreciable amount of the minor protein components is not precipitated under these conditions, it is nevertheless, more nearly representative of the total peanut protein than is either arachin or conarachin, each of which is completely deficient in one of the protein components of the meal.

These results indicate that approximately 99% of the major protein components (A and B) and approximately 58% of the minor protein components of peanut meal are present in the unwashed, undried protein fraction precipitated at pH 4.5.

The conclusions reached above are partially substantiated by the analytical data given in Table III where it will be observed that the protein fraction precipitated at pH 4.5 contains approximately 0.7% sulfur, a figure which is higher than that for arachin (0.4% sulfur) and lower than that for conarachin (1.3% sulfur) (6). Since, as was pointed out previously (6), the minor protein components of peanut meal undoubtedly contain large amounts of sulfur, the protein fraction

TABLE III  
*Analysis of Peanut Protein Fractions<sup>1</sup>*

Fraction Number	Description	Total Nitrogen	Ash	Total Sulfur	Inorganic Sulfur
2	Protein precipitated at pH 4.5	per cent	per cent	per cent	per cent
3	Fraction from mother-liquor of 2	15.66	0.40	0.69	0.03
4	Protein precipitated at pH 6.0	14.08	1.58	1.94	—
5	Protein precipitated at pH 4.5 after removal of 4	17.06	0.50	0.63	0.00
		15.14	1.02	0.88	0.03

<sup>1</sup> Analytical samples were prepared by lyophilization and were subsequently dried for 18 hours *in vacuo* at 50°C. Analyses are reported on a moisture free basis.

precipitated at pH 4.5 would be expected to have a higher sulfur content than arachin which contains none of the minor components, and a lower sulfur content than conarachin which contains a greater proportion of the minor components.

#### *Electrophoretic Analysis of the Mother-Liquor Remaining After Removal of the Peanut Protein Precipitating at pH 4.5*

According to the figures given in Table II for the distribution of the protein components in the protein precipitated at pH 4.5, the mother-liquor obtained after the removal of this fraction should contain only insignificant amounts of the major protein components but should be relatively rich in the minor components.

To verify this conclusion the supernatant solution remaining after the removal of the protein precipitated at pH 4.5 from the extract of 10 g. of peanut meal was dialyzed against distilled water and the precipitate obtained was dissolved in a small volume of ammonia buffer. The solution was dialyzed against ammonia buffer and, after being subjected to a high relative centrifugal force of 3,800  $\times$  gravity to remove a very small amount of suspended material, the solution (approximately 0.2% protein) was examined electrophoretically.

The electrophoretic pattern for the ammonia buffer solution of the protein which remains in the mother-liquor at pH 4.5 is shown in Fig. 2. Only two boundaries having mobilities of approximately - 4.2 and - 2.6  $\times$  10<sup>-5</sup> (Table I), respectively, are apparent and an esti-

mate of the areas under the peaks indicates that the components responsible for these boundaries are present in approximately equal amounts. Since the protein concentration which could be attained in the solution analyzed was necessarily low, accurate measurement of the boundary mobilities and the relative amounts of the two components present cannot be obtained from this pattern. Nevertheless, it is obvious that the concentration of components A and B in this fraction is so small that boundaries for these components are not evident in the electrophoretic pattern. As was mentioned in a previous paper (6), a boundary (designated C) having a mobility of approximately  $-3.5 \times 10^{-5}$  is present in the descending pattern of peanut meal extract. In the ascending patterns for the meal extract, however,

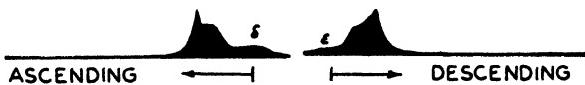


FIG. 2

Electrophoretic Pattern of Peanut Protein Isolated from the pH 4.5 Mother-Liquor After Electrophoresis for 10,620 Seconds in Ammonia Buffer at pH 9.26

the corresponding boundary appeared to be resolved into two peaks. This same behavior is exhibited in the patterns for the protein precipitated at pH 4.5 (Fig. 1). It is likely, therefore, that the two boundaries found in the pattern for the protein fraction which remains in the mother-liquor after precipitation of the major part of the protein at pH 4.5 are identical with those responsible for the composite boundary C of the meal extract and protein patterns. It will be observed that the average mobility of the two boundaries of Fig. 2 is  $-3.4 \times 10^{-5}$ .

The analytical data given in Table III, which indicate that the small fraction of the peanut protein that is soluble at pH 4.5 contains nearly 2% sulfur, serve to substantiate partially the conclusion reached above, namely, that the protein fraction remaining in the mother-liquor is composed predominantly of the minor protein components. It will be recalled that the minor components alone were shown previously (6) to contain almost 3% sulfur. It should be pointed out in this regard that, while the electrophoretic pattern was obtained on the protein fraction which precipitated upon dialysis, the analytical sample was prepared by lyophilization of all that remained in the membrane after dialysis. Inasmuch as the sulfur and nitrogen contents of the

lyophilized fraction are lower than would be expected on the basis of the electrophoretic results, it is apparent that water soluble but non-dialyzable substances which are low in both sulfur and nitrogen are present with the protein in this fraction.

It has been demonstrated (6) that the protein components responsible for the minor boundaries of the meal extract pattern are partially soluble even in 10% sodium chloride solution that has been 85% saturated with ammonium sulfate. The data presented in this section indicate that these components are also partially soluble in aqueous solution at pH 4.5. Suitable modifications of either of these procedures may provide an opportunity to isolate a relatively pure sample of the minor peanut protein components. A method for isolating this fraction will be of interest to those who may continue the investigation of the proteins of the peanut since the high sulfur and methionine content of the minor protein components and possibly the high content of hydroxy amino acids other than threonine and serine may make further investigation of this fraction particularly desirable.

#### *Electrophoretic Analysis of Peanut Protein Precipitated at pH 6.0*

A fraction of the protein contained in peanut meal extract can be obtained by adjusting the extract to pH 6.0. As was mentioned previously, this fraction has physical characteristics which distinguish it from that precipitated within the minimum solubility range of the proteins (pH 4.5) in that the fraction precipitated at pH 6.0 forms as it precipitates a plastic mass which exhibits a marked birefringence and can be pulled manually into fine threads or fibers.

One gram of peanut meal was extracted portionwise with ammonia buffer in the cold and the clear centrifuged extract (50 ml.) was adjusted to pH 6.0 by adding hydrochloric acid. An ammonia buffer solution of the unwashed precipitate was prepared for electrophoretic analysis in the usual manner.

The electrophoretic pattern obtained for this solution is shown in Fig. 3. Since the mobilities of the boundaries present were found to be  $-6.2$ ,  $-5.3$  and  $-3.3 \times 10^{-5}$  (Table I), respectively, it appears that the fraction precipitated at pH 6.0 is very closely similar in composition to the fraction precipitated at pH 4.5. The asymmetry between the ascending and descending patterns discussed above and previously found to occur in the patterns for arachin (6) is also present in Fig. 3. Because of this it is impossible to assign accurate values for the areas

under the A and B peaks of Fig. 3. Area estimations made from the descending pattern of Fig. 3 indicate that components A, B, and the minor components in the protein fraction precipitated at pH 6.0 are present in approximately the same ratio (73, 19 and 8%, respectively) as they occur in the protein fraction precipitated at pH 4.5 (75, 17 and 8%, respectively).

It can be concluded from these data that the plastic properties of the protein fraction precipitated at pH 6.0, as contrasted to the absence of such properties in the protein fraction precipitated at

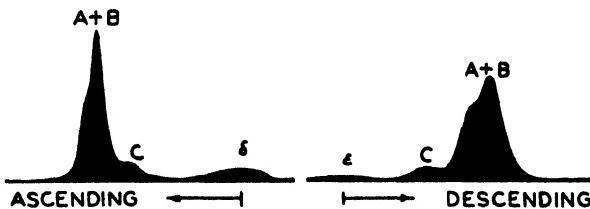


FIG. 3

Electrophoretic Pattern of Peanut Protein Precipitated at pH 6.0 After Electrophoresis for 15,900 Seconds in Ammonia Buffer at pH 9.26

pH 4.5 are not due to qualitative or quantitative differences in protein composition. Since the protein precipitated at pH 6.0 appears to be similar electrophoretically to that precipitated at pH 4.5 the former fraction probably represents only that portion of the total peanut protein which is insoluble at pH 6.0. The unique physical properties of the fraction precipitated at pH 6.0 as compared with that precipitated at pH 4.5 can probably be attributed to the higher net negative electrical charge of the former which may explain the tendency of the proteins composing this fraction to become hydrated and exhibit plastic properties at pH values above their isoelectric points (5).

*Electrophoretic Analysis of the Peanut Protein Fraction Which is Precipitated at pH 4.5 After the Removal of the Protein Precipitable at pH 6.0*

When the mother-liquor which remains after the removal of the protein fraction precipitated at pH 6.0 is adjusted to pH 4.5, practically all of the protein remaining in solution is precipitated. The unwashed precipitate was collected by centrifugation and an ammonia buffer solution containing approximately 0.8% protein was prepared and dialyzed for electrophoretic analysis in the manner described above.

The electrophoretic pattern for the solution is shown in Fig. 4. The mobilities of the components present were found to be  $-6.2$ ,  $-5.2$ , and  $-3.3 \times 10^{-5}$ , respectively (Table I), indicating that all of the meal components are represented in this fraction. Although asymmetry between the ascending and descending patterns exists in this case also, making accurate assignment of area values for the A and B peaks impossible, it appears from the area estimates that the relative proportions of the several components present in this fraction differ from those found in the previously prepared fractions. The amounts of components A, B, and the minor components estimated from the peaks in the descending pattern of Fig. 4 are 34, 36 and 30%, re-

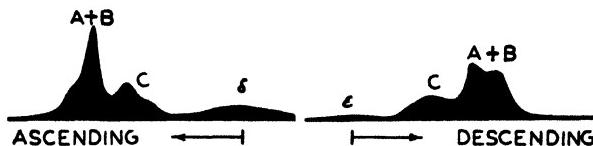


FIG. 4

Electrophoretic Pattern of Peanut Protein Precipitated at pH 4.5 from the pH 6.0 Mother-Liquor After Electrophoresis for 17,100 Seconds in Ammonia Buffer at pH 9.26.

spectively. As would be expected, the analytical results given in Table III indicate that this fraction, which contains relatively large amounts of the minor components, has a higher sulfur content than either the protein fraction precipitated at pH 4.5 or the one precipitated at pH 6.0.

The authors wish to express their appreciation to the Analytical Sections of both the Southern and Eastern Regional Research Laboratories for carrying out the analyses reported in Table III.

#### SUMMARY

The electrophoretic analysis of several protein fractions obtained by simple precipitation procedures from slightly alkaline extracts of peanut meal is presented.

Consideration of the electrophoretic composition and physical properties of these fractions and the high yields with which they can be obtained, strongly suggests the applicability of certain of these fractions in food products or in the preparation of adhesives, fibers and other industrial materials.

A fraction which comprises approximately 90% of the total protein of peanut meal, obtained by adjusting a slightly alkaline extract of peanut meal to pH 4.5, has been shown to contain approximately 99% of the major protein components and approximately 58% of the minor protein components of the meal. Although this fraction is not truly representative of the total protein of the peanut it is, nevertheless, more nearly representative than is either arachin or conarachin, each of which is completely deficient in one of the protein components of the meal.

A protein fraction that is nearly identical electrophoretically with that obtained by precipitation at pH 4.5 can be obtained by precipitation at pH 6.0. This fraction exhibits plastic properties not possessed by the fraction precipitated at pH 4.5.

The protein which remains in solution after the removal of the fraction precipitable at pH 6.0 can be precipitated by adjusting the solution to pH 4.5. The protein fraction thus obtained has a quantitative electrophoretic composition which is markedly different from either of the other fractions described and from arachin and conarachin.

A protein fraction which has a comparatively high sulfur content can be isolated from the mother-liquor remaining after the removal of the protein precipitable at pH 4.5. The protein of this fraction has been shown electrophoretically to be composed almost exclusively of the minor protein components of the peanut and is, accordingly, a readily available source of material for the investigation of these sulfur-rich vegetable proteins.

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# The Determination of Gelatin in the Presence of Plasma Proteins

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Received July 30, 1945

## INTRODUCTION

In connection with a program of research in these Laboratories on the preparation and testing of modified gelatin as a "blood substitute," the need arose for a satisfactory method for estimating gelatin in the presence of plasma proteins. The method described by Waters (1), which involves parallel tungstic acid and trichloroacetic acid precipitations of a given gelatin-plasma protein mixture and the determination of gelatin by difference in the quantities of the precipitates so obtained, has proved upon critical investigation to be subject to errors as great as 100% in the estimation of gelatin and 20% in the estimation of plasma protein in the range of concentrations of these materials encountered in typical retention experiments. These errors may be attributed in great part to the fact that the trichloroacetic acid reagent often does not precipitate the plasma proteins selectively and completely; furthermore, a large error is associated with the determination of a small difference between two large quantities.

It appeared desirable to develop a method which would permit the analysis of a single *entire* precipitate for both plasma protein and gelatin in order to avoid the errors associated with a fractionation procedure. This end could be achieved if a mixed protein precipitate were subjected to two independent analytical procedures, one to provide information about the total protein, and the other to give information about one of the components. The essential absence of the aromatic

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amino acids, tyrosine and tryptophane, from gelatin and their relative abundance in plasma protein (2, 3) proved to be the distinguishing analytical feature which was desired. Thus, if one aliquot of the tungstic acid precipitate of a gelatin-plasma protein mixture is analyzed for total nitrogen by a colorimetric micromethod involving the Nessler reagent and another aliquot is analyzed for total aromatic amino acids by a colorimetric micromethod involving the Folin-Ciocalteu phenol reagent (4), the results can be referred to a calibration curve to give the total plasma protein and, by difference from the total protein, the total gelatin. The method will be illustrated in detail in terms of a calibration experiment.

## EXPERIMENTAL

### *Method*

Citrated rabbit plasma and a solution of decalcified Knox gelatin were adjusted to protein concentrations of 0.926% and 0.940%, respectively, by dilution with 0.85% sodium chloride solution, on the basis of preliminary estimation of nitrogen. These reference solutions were mixed volumetrically to provide suitable solutions ranging from 0 to 1 mg. gelatin per mg. protein. In addition, the two reference solutions and a solution prepared by mixing these in equal parts were each diluted with an equal volume of saline. Each of the solutions was then treated as follows:

*Preparation of the tungstic acid precipitate:* Pipet 1.00 ml. (in quadruplicate for the reference solutions, in triplicate for the others) into a graduated 15-ml. centrifuge tube. Add from a 10-ml. buret 0.25 ml. of 0.67 *N* H<sub>2</sub>SO<sub>4</sub> and mix by rotating. Add, also from a 10-ml. buret, 0.25 ml. of 10% sodium tungstate and mix by rotating, and flipping, if necessary. Allow to stand at least 15 minutes at room temperature. Centrifuge for 15 minutes at approximately 2000 r.p.m. Decant supernatant and allow tube to drain.

*Solution of the precipitate:* To the precipitate add from a buret 1.0 ml. of 1.00 *N* NaOH and dissolve the precipitate with the aid of gentle heating over a low flame. (Precipitates containing more than about 40% gelatin dissolve with difficulty; therefore, they are warmed and allowed to stand several times to complete the process. Complete solution is indicated when no *schlieren*, arising from gradients in the refractive index, appear when a tube which has been allowed to stand for several minutes is gently tapped.) Bring to a volume of 10.0 ml. with distilled water. Cover and mix thoroughly.

*Determination of total protein:* Pipet 1.00 ml. of the solution of the precipitate into a digestion tube calibrated at 35 and 50 ml. Add from a buret 1.0 ml. of concentrated sulfuric acid which has been diluted with an equal volume of distilled water. Digest to dense white fumes over a Meker burner in a hood. (For this purpose we have found it convenient to utilize a circular copper rack supported by a tripod. Each rack can be made to hold 8 or 10 digestion tubes and one analyst can readily manage more than 40 digestions at one time.) Remove from the flame and allow to cool for 30 seconds.

Add one drop of a good grade of nitrogen-free 30% H<sub>2</sub>O<sub>2</sub> directly to the digest and heat again to dense fumes. Remove from the flame and allow to cool for 15 minutes.

Bring the volume to about 30 ml., but not over 34 ml., with distilled water. Add from a buret 1.0 ml. of 5.0 N NaOH and swirl. Add immediately 15.0 ml. of Nessler reagent from a large buret or other suitable volumetric container and swirl. Bring the volume to 50.0 ml. with distilled water and mix by bringing a clean dry footed glass rod up and down several times in the solution. After 15 minutes read the color intensity with a suitable colorimetric instrument. (For this purpose we have found a Klett-Summerson photoelectric colorimeter satisfactory.)

Subtract from this reading the reading given by a blank in which the protein is omitted. A standard may be prepared by adding to a blank, before or after digestion, 1.00 ml. of a standard solution of ammonium sulfate containing 0.100 mg. nitrogen per ml. (It has been our practice to include one or more blanks with each set of 8 or 10 analyses and to include sufficient standards to establish the standard value adequately.)

The amount of protein in the analyzed aliquot is obtained from the relation

$$\text{mg. protein} = \frac{\text{unknown reading}}{\text{standard reading}} \times 0.1 \times 6.25$$

The use of the customary conversion factor 6.25 for both plasma protein and gelatin introduces no uncertainties into the determination of the proportional amount of gelatin retained at a given time.

*Determination of the phenol color:* With the same pipet used in the total protein determination, pipet 1.00 ml. of the solution of the tungstic acid precipitate into a calibrated 15-ml. centrifuge tube. From this point the analysis is conducted exactly as described by Pressman (4) except that the volume is brought only to 6.0 ml. after the addition of alkali and is adjusted to 7.5 ml. after digestion and cooling. The color intensity is recorded in the arbitrary units of the colorimeter scale.

### Results

It was reasonable to expect that the intensity of phenol color referred to unit weight of a given protein would not be a constant but would depend on the concentration of protein in the sample subjected to analysis. This is so because the relation between phenol color and weight of protein is not linear, but is represented rather by a curve which rises steeply from the origin, the slope decreasing gradually and then becoming constant at about 200 γ of protein (4). This non-proportional production of color was verified in the present experiments. The three half-concentrated solutions gave higher unit phenol color production than the corresponding undiluted solutions. This situation is important analytically because of the variations in protein concentration which occur in the usual retention experiments. It was therefore necessary to

find some way of expressing the color-weight relationship which would not depend on the concentration of protein.

It was observed that, for both plasma protein and gelatin, plots of phenol color against weight of protein had a common extrapolated intercept, which was found to be 33. Furthermore, the slopes of these plots could also be obtained and it is possible to write the following equations which relate the variables in the linear portion of the color-weight curve:

$$(I) \quad C = 33 + 42G,$$

and

$$(II) \quad C = 33 + 432P$$

where  $C$  is the color, expressed in the arbitrary units of the colorimeter scale,  $G$  is the aliquot weight of gelatin in mg., and  $P$  is the aliquot weight of plasma protein in mg. The common intercept allows us to write the following general equation for the production of color by a gelatin-plasma protein mixture:

$$(III) \quad \begin{aligned} C &= 33 + 42(G + P) + (432 - 42)P \\ &= 33 + 42T + 390P \end{aligned}$$

where  $T$  is the aliquot weight of total protein in mg. Since we can determine  $C$  and  $T$  experimentally, we can find  $P$  from Equation (III) and  $G$  from the difference  $T - P$ . The constants of Equation (III) are functions not only of the proteins involved but also of the colorimetric apparatus and *must, therefore, be determined for each new system*.

If the general relation is valid, a plot of the corrected phenol color,  $C'$ , which is made equal to  $C - 33 - 42T$ , against  $P$  should be a straight line passing through the origin; furthermore, this line should have a slope of 390 and should represent the data for the half-concentrated as well as the undiluted samples. Fig. 1 is a complete confirmation of these statements. The abscissae for this plot were calculated from the mixing ratio and the Nessler values for the reference solutions and for the mixtures.

It is convenient to use such a plot for obtaining values of  $P$  graphically from the corrected color reading. The plot may not be used if the total protein concentration is below the point where the slope of the curve of uncorrected color against weight of protein (4) becomes linear. A five-fold dilution of citrated plasma gives approximately 1% solutions of protein, which are suitable for analysis.

We have been able to recalculate the data obtained in two entirely independent experiments involving 13 gelatin-plasma protein mixtures; when suitable corrections were made for the difference in the color filters used, it was found that these data provided additional verification for the relation expressed in Equation (III).

### DISCUSSION

The method described above possesses the following technical advantages:

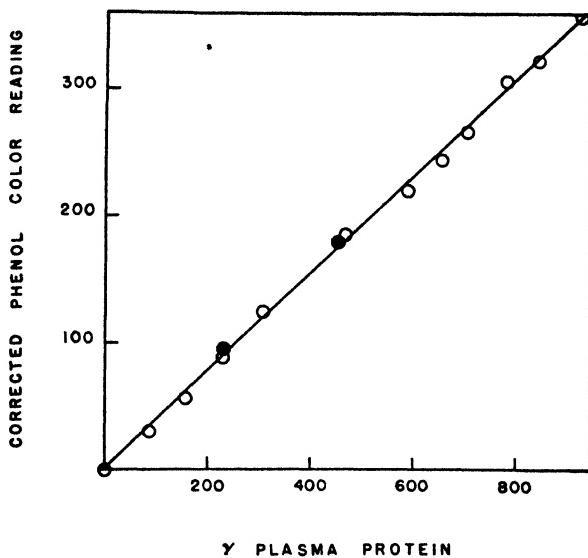


FIG. 1

A Calibration Curve for Obtaining the Weight of Plasma Protein  
from the Corrected Phenol Color Reading

○ samples at approx. 1% protein; ● samples at approx. 0.5% protein.

1. No fractionation of gelatin and plasma protein is required; instead a single *total* precipitation is carried out. The errors arising from the co-precipitation of gelatin and plasma protein, as, for example, in the trichloroacetic acid fractionation of a mixture, are thereby avoided.
2. The analytical operations are considerably reduced by the formation and handling of a single precipitate.

3. Errors of pipetting and diluting made up to the point of sampling the solution of the precipitate do not affect the determination of the composition of the precipitate, but only of the quantity. Furthermore, since aliquots for phenol color and for nitrogen analyses may be taken with a single pipet, errors in the calibration of a given pipet likewise affect the determination only of the quantity of a precipitate.

The total protein and phenol color determinations are subject to average errors of  $\pm 0.5\%$  and  $\pm 1\%$ , respectively, when a single pipet is used for sampling all of the solutions of the tungstic acid precipitates prepared from a given mixture. When different pipets are used for sampling the *original* gelatin-plasma protein mixture, the total protein determination is subject to an average error of approximately  $\pm 1.5\%$ . Under these conditions the phenol color determination is expected to be subjected to a similar error, although our data do not permit this calculation. The errors in the estimation of the quantities of gelatin and plasma protein in the original mixtures were found, by a comparison of experimental with calculated values, to be approximately 5% and 3%, respectively. These errors, obtained by averaging the deviations of experimental from calculated values for all the mixtures, represent total errors for the method. The figures given support the contention that the method proposed in this paper is suited to the study of the retention of gelatin in the mammalian circulation.

The method is subject to the limitation imposed by the possibility that the composition of the plasma protein in terms of its protein individuals, and therefore the unit content of aromatic amino acids, changes in the course of a retention experiment. Adequate information is lacking, at present, for analyzing this limitation in detail. The results reported by Minot and Keller (5), however, for the proteins of human serum suggest that the limitation may not be a serious one in this case. These workers found tyrosine factors of 13.25, 13.43, and 12.93 for the total serum proteins, albumins and globulins, respectively.

The method appears to be ideally suited to the analysis of a mixture of two pure proteins, the analytical properties of which permit sufficient resolution between two mixtures of slightly different composition, and may be useful, as well, in the study of complex-formation between two such proteins. It is believed that further quantitative refinements may significantly increase the utility of the method in studies of pure proteins.

### ACKNOWLEDGMENTS

The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the California Institute of Technology.

The authors wish to acknowledge the help received from other members of the group working on the blood substitute problem, namely, Professor Linus Pauling (Responsible Investigator), Professor Dan H. Campbell, Dr. Norman Abrahamsen, Dr. David Pressman and Mary L. Sease. In addition, the authors wish to thank Dr. Verner Schomaker for discussing the results and Mr. Dan Rice for drawing the figure and assisting with some of the analyses.

### SUMMARY

A method is described for the determination of gelatin in the presence of plasma protein, based on the differential reaction of the Folin-Ciocaltéu phenol reagent with these materials.

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# Lipoxidase

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Received August 2, 1945

## INTRODUCTION

An enzyme which peroxidizes unsaturated fats and which bleaches carotenoids was discovered in the soy bean by Bohn and Haas (1) in 1928 and has been used since that time for the commercial bleaching of wheat flour. It was discovered independently by André and Hou (2) in 1932 and named lipoxidase. Sumner and Sumner (3) showed in 1940 that this enzyme does not act upon carotenoids directly, but by an induced reaction in which some intermediate fat oxidation product is the effective agent.

Of the more recent papers dealing with lipoxidase, one of the most important is by Balls, Axelrod and Kies (4). These authors have purified soy bean lipoxidase to a considerable extent and have shown that it does not peroxidize pure oleic acid. They state that lipoxidase does not occur in animals, but it should be noted that Banks (5, 6) has found this, or a similar enzyme, in herring muscle; and Lea (7) has found a fat-peroxidizing enzyme in the adipose tissue and muscle of the pig. Hove (8) has demonstrated the *in vitro* destruction of carotene by water extracts of minced rat stomachs in the presence of methyl linoleate. He found his enzyme essentially comparable in properties to soy bean lipoxidase.

Balls *et al.* claim crude preparations of lipoxidase are accompanied by a thermostable substance which increases the catalytic action of lipoxidase upon unsaturated fats. Their pure "enzyme protein" had slight activity alone. The residual activity was attributed to the incomplete removal of the activator during purification. By using the filtrate from boiled suspensions of soy bean meal, or by using the concentrated and purified activator which Balls *et al.* describe, and following the precaution of adding the activating material either to the enzyme or to the fat suspension before the addition of the enzyme, we have been unable to repeat their method for activating lipoxidase. Further, we have employed a variety of procedures for the purpose of splitting lipoxidase reversibly into apoenzyme and coenzyme, but without success. These authors also say that the activator occurs in barley malt. We find, on the contrary, that lipoxidase itself is present in moderate concentration in green barley malt. Boiled malt has no activating effect. In short, then, we have found no activator for lipoxidase.

There appears to be no satisfactory method for determining lipoxidase activity. In the Renner method (9), where one analyzes for the peroxide formed, the enzyme activity falls off rapidly, presumably because the lipoxidase is inactivated by the rapid stirring. When one follows lipoxidase activity with the Warburg apparatus, it is found that there is no simple relation between enzyme action and the consumption of oxygen, at least over a range where convenient enzyme concentrations are employed. Recent investigators have adapted the qualitative carotene decolorization test (10) for quantitative work. Reiser and Fraps (11) have devised a method for determining the quantity of "carotene oxidase" present in legumes based on the per cent carotene destroyed. Here carotene-Wesson oil substrate is acted upon by the enzyme for 30 minutes at 35°C. and pH 6.5. The residual carotene is extracted with ether and read in a photoelectric colorimeter. From a blank determination the initial carotene in the ether extract is read. These two readings enable one to calculate the amount destroyed. Ten units are defined as that quantity of enzyme which oxidizes 50% of the carotene in 30 minutes and under their prescribed conditions. Balls *et al.* have also made use of the qualitative carotene decolorization test in their assay of lipoxidase activity. They determine the time required for lipoxidase, acting upon ethyl linoleate, to induce the oxidation of carotene. The end point is where the colorimeter shows a 30% decrease in the concentration of carotene. We have employed this method in modified form, using the easily prepared soy bean fatty acids, instead of ethyl linoleate. Our carotene suspensions were stabilized by the addition of gum arabic in phosphate buffer of pH 6.5. The carotene decolorization method, as Balls *et al.* have already noted, possesses the serious disadvantage that the rate of carotene oxidation is parallel to the lipoxidase concentration only over a very narrow range. Hence, the carotene units must be defined in seconds required for the carotene to undergo decolorization, as well as in other terms.

Finally we have been able to purify soy bean lipoxidase about 60-fold.

## EXPERIMENTAL

### *Purification of Soy Bean Lipoxidase*

One hundred grams of potassium ammonium alum is dissolved in cold water and sodium hydroxide is added to bring the pH to 6.5. The final volume is made up to 1000 ml. with ice cold distilled water. To 100 g. of defatted and sifted soy bean meal in a large beaker, the potassium ammonium alum-water is added with stirring by

means of a wooden paddle. The mixture is filtered in the ice chest overnight as the mixture does not centrifuge well. Enough neutral saturated ammonium sulfate is added to the filtrate to make the solution 66% saturated. The precipitate is centrifuged off in the cold room and then dissolved in 0.5 M phosphate buffer of pH 6.5. The enzyme solution is then made 25% saturated with neutral, saturated ammonium sulfate and the precipitate removed by centrifugation. The supernatant contains the enzyme; it has a greenish-yellow fluorescence in ultra-violet light. The enzyme is dialyzed in the ice chest against dilute neutral phosphate buffer in cold distilled water for four hours, the outer dialysis water being changed repeatedly. The loss in activity during this period of dialysis is not appreciable. Any protein which precipitates out is removed by centrifugation and discarded. The supernatant solution contains the enzyme purified to the extent of about 60-fold over a 2% water extract of defatted soy bean meal, based on protein nitrogen. The enzyme solution can be purified further by addition of neutral ammonium sulfate to make the solution 40% saturated. This precipitates inactive protein.

#### *Determination of Lipoxidase Activity*

Place in a clean dry 250 ml. Erlenmeyer flask 5 ml. of stock carotene solution. Add 1 ml. of neutralized fatty acids of soy bean. Mix and add 100 ml. of distilled water which contains 20 ml. of gum arabic-phosphate buffer of pH 6.5. Mix by swirling the flask. An even suspension of carotene is thus obtained. Using a blue filter, read in a photoelectric colorimeter to obtain an initial value. To this suspension add from 0.1 to 1 ml. of properly diluted enzyme solution. Mix and place some of the solution in the colorimeter tube. Make readings from time to time to ascertain how many seconds are necessary for the enzyme to destroy 50% of the carotene, as shown by the colorimeter reading decreasing from the initial reading by 50%. Now run other determinations, using appropriate quantities of enzyme solution. Having obtained some 3 or more values, plot the enzyme volumes against times of 50% decolorization. Note what volume of enzyme has decolorized the carotene 50% in 300 seconds. This volume contains 1 unit of lipoxidase. Fig. 1 illustrates a typical curve obtained using this method of assay.

The purity of lipoxidase is based on the total protein as determined by the method of Robinson and Hogden (12).

#### *Preparation of Reagents*

**Stock Solution of Carotene:** Two hundred mg. of crystalline carotene (90%  $\alpha$  and 10%  $\beta$ ) are stirred with 1500 ml. of redistilled acetone. Five hundred ml. of alcohol are added and the solution shaken and warmed to dissolve the carotene. The undissolved carotene is filtered off. About 150 mg. of the carotene dissolves. Five ml. of this carotene solution, when used as described in the method of assay, reads 60 ini-

tially in the Fisher photoelectric colorimeter, using a blue filter and scale A. The end point is taken when the reading becomes 30.

*Soy Bean Fatty Acids for Substrate:* The soy bean fatty acids are prepared by boiling 50 g. of soya oil with alcoholic sodium hydroxide. The alcohol is then boiled off and water added. The fatty acids are precipitated by addition of sulfuric acid, washed with hot water, and kept in the ice chest until used. The fatty acids used in the assay are prepared by pipetting 1 ml. of the melted material into 100 ml. of alcohol, and then neutralizing with  $N$  NaOH, using phenolphthalein as indicator. The final volume is made up to 300 ml. with alcohol.

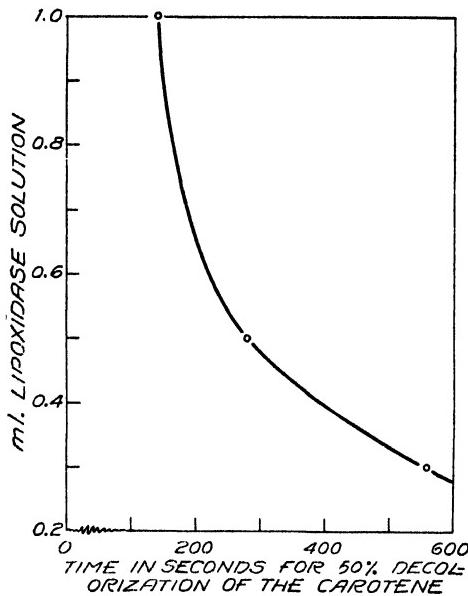


FIG. 1

*Gum Arabic-Phosphate Buffer:* Forty g. of gum arabic are heated in 500 ml. of distilled water over a steam bath until dissolved and then brought to about pH 6.5 by adding approximately 20 ml. of 0.1  $N$  NaOH. Then 200 ml. 0.5M phosphate buffer of pH 6.5 are added and the suspension of tri-calcium phosphate formed filtered off overnight. The filtrate is diluted to two liters and a few drops of toluene added. The gum arabic-phosphate buffer is preserved in the ice chest. The phosphate buffer is 60 ml. of 0.5 M  $KH_2PO_4$  to 40 ml. of 0.5 M  $Na_2HPO_4$ .

#### CONCERNING THE ACTIVATOR

Table I shows no activation of lipoxidase with either the crude activator or the purified activator described by Balls *et al.* Neither does

TABLE I

Enzyme ml.	Activator ml.	Time of incubation min.	Time for decolori- zation to a reading of 30 min.
1	—	—	6
—	1 (Crude activator)	—	No decolorization
1	1 "	—	6½
1	1 "	8	6½
1	1 "	15	9
1	0.5 "	—	6½
1	0.5 "	10	6½
1	2 "	—	7½
1	2 "	10	8
—	1 (Purified activator)*	—	No decolorization
1	1 "	—	7
1	1 "	10	7½

\* A few mg. of the purified and concentrated activator as described by Balls *et al.* was dissolved in distilled water and neutralized to phenol red.

incubation of the enzyme with these substances effect any activation. An apparent inhibition was observed at times due possibly to inactivation of the lipoxidase during the period of standing with the activator. At times we noted an activating effect on lipoxidase using the crude activator; but in such cases, we found the crude activator was itself capable of lipoxidase action. Further heating destroyed the activating effect of the crude preparation. The enzyme used in our experiments was prepared as outlined above and was so diluted that one ml. had about one of our units.

The crude activator was prepared by mixing 5 g. of defatted soy bean meal with 100 ml. of glass distilled water and 2 ml. of 1.08 N acetic acid. The suspension was strained through cheese cloth and heated to 95°C. for 10 minutes. The supernatant after centrifugation was the crude activator and contained 2.2 mg. of protein per ml.

Using lipoxidase solutions which had lost activity upon long dialysis or the protein fraction from pure enzyme preparations, we could not demonstrate any activation of the enzyme. We were also unable to obtain any "enzyme protein" which was void of lipoxidase activity and which became active when the activator of Balls *et al.* was added.

We have observed that digestion with commercial trypsin decreased lipoxidase activity, while digestion with crystalline trypsin was without effect. Hence, at the present time it appears to us that the profitable

investigation of lipoxidase awaits either the crystallization of this enzyme in pure form, or else the discovery of some substrate which possesses a simple structure. In this connection we wish to report that we have synthesized sorbic acid,  $\text{CH}_3\cdot\text{CH}:\text{CH}\cdot\text{CH}:\text{CH}\cdot\text{COOH}$ , but find that lipoxidase does not peroxidize this compound. Recently Holman and Burr (13) have observed that linoleic, linolenic, and arachidonic acids, after action of lipoxidase, are converted into substances, apparently conjugated, unsaturated carbonyl compounds, which possess a strong absorption in the ultra violet region. It seems possible that the spectrophotometric measurement of this change can be used in testing for lipoxidase activity.

We wish to express our thanks to the Rockefeller Foundation for financial support.

### SUMMARY

A method for the purification of soy bean lipoxidase is described by means of which the enzyme is concentrated 60-fold.

An adaptation of the qualitative carotene decolorization test for lipoxidase assay is described.

Contrary to the findings of Balls *et al.*, our investigations have revealed no activator for lipoxidase.

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# Thermal Destruction of Influenza A Virus Hemagglutinin.

## I. The Kinetic Process<sup>1</sup>

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Received August 7, 1945

### INTRODUCTION

In 1941 it was discovered by Hirst (1) and independently by McClelland and Hare (2) that chicken red blood cells are agglutinated when they are immersed in the allantoic fluid of chicken embryos infected with influenza virus. It was shown first by McClelland and Hare and later by Hirst (3) that the red cells adsorb the agglutinin and the virus infectivity. Hirst showed that the adsorbed activities could be eluted from the red cells readily by simply warming the suspension to 37°C. The results of McClelland and Hare first indicated that the agglutinating activity is proportional to the mouse infectivity titer, and those of Hirst (4) showed conclusively that this is so.

Friedewald and Pickels (5) demonstrated by means of sedimentation experiments carried out in sucrose gradients in an angle centrifuge that the sedimentation rates of the infectious principle and of the hemagglutinin were roughly the same. They concluded that both activities were associated with particles at least 60 m $\mu$  in diameter. In somewhat similar experiments carried out by Sharp *et al.* (6), Taylor *et al.* (7), and Stanley (8), essentially the same results were obtained. Stanley carried the matter further by showing that the specific agglutinating activity and infectivity of the component of chicken embryos having a diameter of 115 m $\mu$  were much higher than those of smaller particles. More recently, Lauffer and Miller (9), through studies involving the use of the separation cell in the ultracentrifuge, showed with a relatively high degree of precision that the sedimentation rates of the 115 m $\mu$  spherical particles and of the red blood cell agglutinating activity are indistinguishable. It was also shown that, in virus preparations containing aggregates of the 115 m $\mu$  particles, an amount of agglutinin comparable to the amount of aggregated material sedimented

<sup>1</sup> The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pittsburgh. It was also supported in part by a grant from the Buhl Foundation to the University of Pittsburgh.

more rapidly than the  $115 \text{ m}\mu$  component. It was found, further, that within the limits of accuracy of the tests, chicken embryo infectivity and mouse infectivity sedimented at rates indistinguishable from that of the agglutinin. Miller, Lauffer, and Stanley (10) showed that the agglutinin migrates with the  $115 \text{ m}\mu$  particles in the electrophoresis apparatus. Finally, Knight (11) recently isolated a highly purified influenza A virus preparation from the lungs of diseased mice and found that the chicken red blood cell agglutinating activity, the infectivity, and the physical properties of the preparation were indistinguishable from those of preparations obtained from diseased chicken embryos. These facts lead to the conclusion that the red blood cell agglutinating factor is intimately associated with the infectivity and that both are borne by the  $115 \text{ m}\mu$  spheres characterized in detail by Lauffer and Stanley (12). Therefore, the agglutinating ability is an integral property of the influenza virus particle and merits study as such.

Several observations on the stability of the agglutinin have been recorded. Hirst (4) reported that, under some circumstances, agglutinating activity remained in influenza virus preparations which had lost their infectivity. Miller (13) confirmed this observation by showing that when influenza virus is stored at  $4^\circ\text{C}$ . in buffers more alkaline than pH 7 the infectivity decreases more rapidly than the agglutinating activity. Miller also found that the agglutinating activity was more stable in concentrated virus preparations than in dilute ones, and more stable at  $4^\circ\text{C}$ . than at room temperature.

In the present study the destruction of the hemagglutinin of PR 8 influenza A virus was studied at elevated temperatures. This paper describes results related to the nature of the kinetic process involved and the effect of virus concentration. A subsequent paper will deal with the effect of varying pH.

#### MATERIALS AND METHODS

*PR 8 Influenza A Virus:* The influenza A virus of the PR 8 strain was obtained from Dr. W. M. Stanley <sup>2</sup> of the Rockefeller Institute for Medical Research, Princeton, N. J. The previous history of the strain was described by Stanley (14). It was brought to this laboratory in the form of frozen allantoic fluid held at about  $-70^\circ\text{C}$ . It was passed once in 10 day chicken embryos and was stored as allantoic fluid at about  $-70^\circ\text{C}$ . in 1 cc. ampules.

*Purified Virus Preparations:* White leghorn chicken embryos which had been held at  $39^\circ\text{C}$ . for 10 days were inoculated by way of the allantoic cavity with 0.2 cc. portions of the stock inoculum diluted a million fold with 0.1 *M* phosphate buffer at pH 7. They were then incubated for two days at  $36^\circ\text{C}$ . After that, they were held at about  $4^\circ\text{C}$ . for one day and, finally, the allantoic fluid was harvested by rupturing the allantois and inverting the embryo. The pooled allantoic fluid was then centrifuged at a speed of about 5000 r.p.m. in an angle centrifuge to remove large particles. The

<sup>2</sup>The authors wish to express their appreciation to Dr. Stanley for supplying the original virus material.

precipitate was discarded and the supernatant fluid was passed at a rate of 15 cc. per minute through a Sharples supercentrifuge operated at between 45,000 and 50,000 r.p.m. The supercentrifuge was cooled by coils connected to a small refrigeration unit. In most instances, the virus adhered to the walls of the centrifuge bowl. It was suspended in a small volume of 0.1 M phosphate buffer at pH 7. Finally, this suspension was centrifuged at 5000 r.p.m. in an angle centrifuge to remove aggregated material and other large particles. The final concentration of virus was usually between a hundred and a thousand times that of the allantoic fluid.

*Chicken Red Cells:* Red cells were obtained from freshly drawn citrated blood of adult chickens. They were prepared as described by Hirst and Pickels (15) and were stored in 30% to 80% suspensions in physiological saline. Fresh supplies of red cells were received at least once a week.

*Agglutinin Titrations:* The unit of hemagglutinating activity was defined by Hirst and Pickels (15) as the amount per cc. which will agglutinate half of the red cells from a 0.75% by volume suspension. The hemagglutinin titrations were carried out by determining the dilution corresponding to unit concentration essentially as described by Hirst and Pickels. However, a commercially available Fisher photoelectric colorimeter, equipped with a special brass adapter to hold 10 × 75 mm. test tubes, was used for recording the degree of agglutination in each tube. When a red filter was used with this instrument, a 0.37% suspension of red cells gave a reading of about 35.

The two-fold serial dilutions were made according to the following schemes. For virus samples of highest agglutinating activity, 0.10 cc. of virus was transferred to 9.9 cc. of a physiological saline solution containing 0.01 M phosphate buffer at pH 7. This represented a relative concentration of virus of 1/100. Solutions containing relative concentrations varying by a two fold factor between 1/200 and 1/6400, inclusive, were prepared each by single step dilutions of this 1/100 solution. Then 1 cc. of a 1.5% suspension of red cells was added to 1 cc. of each dilution to give final relative concentrations of virus ranging from 1/200 to 1/12,800. For virus samples of lower agglutinating activity, the first stage dilution was 0.10 cc. of virus to 2.4 cc. of solvent to give a relative concentration of 1/25. Solutions with relative concentration between 1/50 and 1/800, inclusive, were then prepared each by single step dilutions of this 1/25 solution. Finally, equal volumes of red cell suspension were added to each. The advantage of this dilution procedure is that systematic errors are not accumulated as in the case of successive two fold dilutions. The method of successive two fold dilution was used only in cases where the agglutinating activity was too low to permit the use of the direct dilution method.

*Inactivation Experiments:* The virus was inactivated by placing it in a 10 × 75 mm. test tube immersed in a water bath capable of holding the temperature constant to within ±0.1°C. During an experiment, something less than a cubic centimeter of virus was heated at one time, and 0.10 cc. samples were withdrawn periodically for analysis.

## DISCUSSION OF EXPERIMENTAL RESULTS

*Accuracy of Agglutinin Titrations:* Miller and Stanley (16) studied the accuracy of the agglutinin titration reaction in considerable detail

and found that, in any particular case where duplicate titrations were made upon different samples, the chances were 19 out of 20 that differences between the mean end points of 8.4% were significant. We have carried out a single experiment to illustrate in a slightly different way the accuracy of this titration. A series of six dilutions of a virus sample was prepared, each half as concentrated as the preceding one. Activity titrations were then carried out on each. The results are shown in Fig. 1

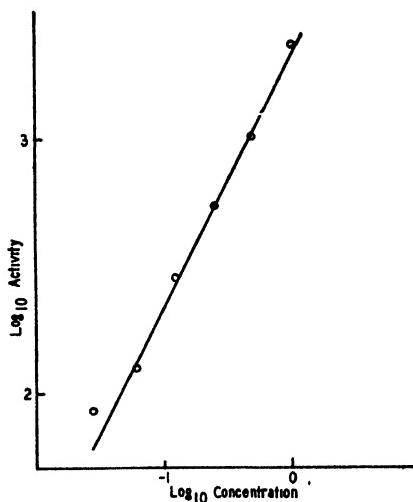


FIG. 1

Activity Titration of PR-8 Influenza A Virus at Several  
Known Relative Concentrations

Activity is expressed as the number of units of agglutinin per cc.

where the log. of activity is plotted against the log. of relative concentration. The data fit a straight line with a slope of unity, as they should, if the titration is reliable over a range of concentrations.

*Change of Concentration With Time of Heating:* In dealing with the inactivation of a virus, one normally attempts to apply the equation of a first order reaction to the data representing the change in activity with time. This is done because the inactivations of tobacco mosaic and other viruses have been shown by Price (17) to follow with high fidelity the courses of first order reactions. In contrast with these results, Bourdillon (18) reported that the thermal inactivation of the

SK strain of poliomyelitis virus did not follow the first order law. In approximately sixty to seventy experiments, carried out in the present study on the thermal destruction of the hemagglutinin of PR-8 influenza A virus, the law of a simple first order process was not obeyed. When the logs. of residual agglutinating activities were plotted against times of heating, in the majority of the cases, one was not able to fit a straight line to the data. In general, curves with decreasing negative slopes were obtained. However, when the reciprocals of the square roots of the residual concentrations were plotted against the times, it was generally found that straight lines could be fitted to the data. This situation is illustrated by the data in Fig. 2. These data were ob-

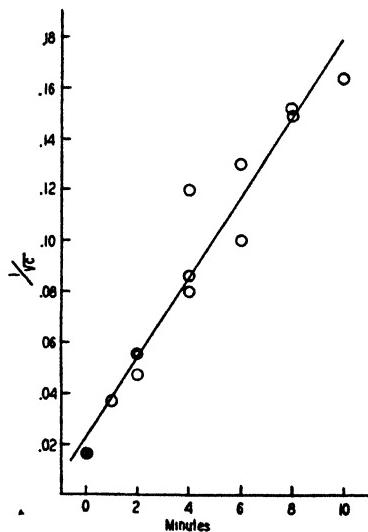


FIG. 2

The Course of the Destruction at 61°C. of the Hemagglutinin of PR-8 Influenza A Virus in a Phosphate Buffer of Ionic Strength 0.20 and at pH 6.9  
The reciprocal of the square root of agglutinating activity is plotted against time in minutes.

tained on three different occasions by heating at 61.0°C. samples of the PR-8 influenza A virus preparation dissolved in potassium phosphate buffer with an ionic strength of 0.20 and a pH of 6.9.

It may be seen that the data represent a reasonably good fit to a straight line. In order to evaluate objectively the relative goodness of

the fit of these data to a straight line, as compared to the cases wherein the log. of concentration and the reciprocal of concentration are plotted against time, the statistical method described by Lauffer and Price (19) was used.

In essence, this method consists of finding the best fitting straight line for each set of data by the method of least squares, followed by a calculation of a statistic, the standard error of estimate, which measures the dispersal of the data about that line. The ratio of the slope of the line thus fitted to the standard error of estimate is called the tau value. The higher the tau value, the better is the fit of the data to the straight line in question. Tau values of 0.64, 2.26 and 0.50, respectively, were calculated for the data of Fig. 2 for the cases in which the log. of concentration, the reciprocal of the square root of concentration, and the reciprocal of concentration were considered as linear functions of time. This result demonstrates conclusively that the second procedure gives by far the best representation of the data.

The relationship shown above usually seems to hold for the first 90% to 99% of the course of the reaction, but many cases of deviation have been observed in reactions which were allowed to proceed more nearly to completion.

It was thought at first that some lack of control in the experiments was the cause of this unusual apparent kinetic behavior. Three possible sources of error were investigated. First, it was reasoned that the buffer might lose a small amount of carbon dioxide during heating and thus change in pH during the course of the reaction. To test this idea, a phosphate buffer at pH 6.9 was made up with extreme precautions to prevent the inclusion of carbon dioxide, and the virus equilibrated against it was first boiled under vacuum at room temperature to expel any carbon dioxide in it. When inactivation experiments were carried out with this virus-buffer system, the results were the same as usual. Thus the loss of carbon dioxide did not seem to be the cause of the anomaly. It was next supposed that virus heated in contact with atmospheric oxygen might change in such a way as to slow up the hemagglutinin inactivation. To test this idea, some of the virus-buffer system just described was heated under carefully washed mineral oil. The result was the same as usual, thereby dispelling that possibility. Finally, it was thought that perhaps the end products of the reaction were in some way interfering with the measurement of agglutinating activity. To test this idea, a portion of virus preparation was heated at 90°C. for fifteen minutes and cooled. Then the heated virus was mixed with unheated virus in various proportions, and agglutinin activity was determined on each mixture. The results are shown in Fig. 3. There is no evidence that

the presence of inactivated virus affects the measured activity of the unchanged virus.

The meaning of the uncommon kinetic process is a little difficult to understand. An equation in which the reciprocal of the square root of concentration is a linear function of time is the integrated form of an equation in which the derivative of concentration with respect to time is proportional to the three halves power of concentration. The fact that the inactivation of the hemagglutinin follows the course of this integrated equation would suggest that this reaction is one of the three

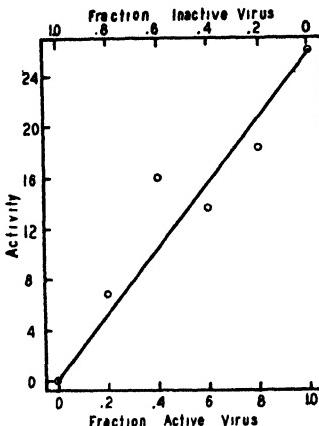


FIG. 3

Agglutinin Activity of Various Mixtures of Virus Heated at 90°C.  
for 15 Minutes, and Unheated Virus  
Activities are expressed as hemagglutinin units divided by 100

halves order, if the analogy with reactions of the first and of the second order is to be indulged. Indeed, there are chemical reactions of the three halves order. For example, Farkas (20) has shown that the high temperature conversion of para hydrogen to ortho hydrogen is a reaction of the three halves order. Actually, it is a bimolecular reaction between an atom and a molecule. If the analogy is to be carried over to the destruction of hemagglutinin, one would have to suppose that the reaction was bimolecular between whole virus particles and half particles. Needless to say, such a mechanism does not seem reasonable.

The hemagglutinin is an integral part of the influenza virus particle, a spherical body on the average 115 m $\mu$  in diameter. On *a priori* grounds,

the destruction of any attribute of such a large particle ought to be a unimolecular process, for the probability of collisions of such large objects is so small that the reaction rates of bimolecular processes would be essentially zero. Thus, the destruction of hemagglutinin ought to be unimolecular, at least with respect to influenza virus. The apparent three halves order, then, ought to be merely the end result of some complication in the kinetic process. It is possible to eliminate certain types of complications from a consideration of the nature of the data observed.

Where the destruction of the hemagglutinin is considered as a first order process, the data obtained invariably indicate that the specific reaction rate is greater during the early stages of the reaction than during later stages. There are several kinds of complications commonly met in the study of reaction kinetics. Often, an over all reaction is the result of a series of reactions,  $A \rightarrow B \rightarrow C$ , etc. If all of the steps are first order, and if one reaction proceeds much more slowly than any of the others, the over all reaction still appears to be first order. However, if two or more of the intermediate reactions appear to have specific reaction rates of the same order of magnitude, the over all process deviates from the first order law. In this case, the over all reaction rates will appear to be too slow in the early stages of the reaction. Thus, this sort of mechanism can not account for the results obtained with the hemagglutinin. A second type of disturbance could be due to the existence of parallel reactions of the first order, each leading in its own way to inactivation. However, when a process of this sort is followed by measuring the disappearance of the starting material, the over all reaction still appears to be of the first order. Therefore, this possibility is eliminated. A third possibility is that the influenza virus particles are inhomogeneous with respect to the ease with which hemagglutinin can be destroyed. Thus, if at a given temperature some particles have a higher specific reaction rate than others for the destruction of hemagglutinin, the over all disappearance of activity, when viewed as a first order process, would seem to proceed more rapidly in the early stages when the fast reacting components were still present in appreciable concentration, and less rapidly in the later stages when only the slow reacting components were left in appreciable concentration. This sort of mechanism does agree with the observed results in the case of the destruction of hemagglutinin. The assumption of inhomogeneity with respect to stability of influenza virus is not unreasonable, for the virus

preparations have already been demonstrated to be somewhat inhomogeneous with respect to size (12). Experiments to test this assumption are being undertaken and will be reported later if successful.

Whatever the cause of the apparent kinetic process, use can be made of it for the determination of a constant descriptive of the reaction rate in the early stages of the reaction. In other words, the relationship between the reciprocal of the square root of activity and time provides at least a useful method of interpolating data and, therefore, of calculating a rate inversely related to the time of half life. The relationship obtained is characteristic of a reaction whose rate at any particular moment is proportional to the three halves power of the residual concentration. The proportionality constant, known as the specific reaction rate, can be shown by integration to be equal to twice the slope of the straight line obtained when the reciprocal of the square root of concentration is plotted against time. The constant so calculated has the dimensions of the reciprocal of seconds times square root of concentration. Thus, the magnitude of the constant depends upon the unit of concentration chosen. In order to compare reaction velocities, therefore, it is necessary to employ a standard unit of red cell agglutinating activity.

*The Effect of Initial Virus Concentration:* It has been observed in several instances that the rates of inactivation and denaturation of viruses vary in an inverse manner with concentrations. Price (17) found that the rate of inactivation of tobacco mosaic virus was higher in initially dilute solutions than in initially more concentrated ones. The same thing has been found to be true for the denaturation of tobacco mosaic virus by heat (19) and by urea (21). Miller (13) observed that the stability of the hemagglutinin of influenza virus at 4° and at 23° seemed to be greater in initially more concentrated solutions. All of these results suggested that the inactivation of the hemagglutinin at high temperatures be studied at several virus concentrations. To that end, a sample of the virus preparation was equilibrated against a buffer, and then aliquots were inactivated at 61°C. at full concentration, at a  $\sqrt{10}$ -fold dilution, and at a 10-fold dilution with the buffer. Specific reaction rates of .014, .031 and .063, respectively, were obtained. Thus, the effect, common to several virus inactivations, of initial virus concentration upon specific reaction rates has been found to apply equally to the thermal destruction of the agglutinin of influenza virus.

## SUMMARY

The thermal destruction of the hemagglutinin of PR-8 influenza A virus seems to follow the course of a reaction of the three halves order. The correctness of this conclusion was evaluated objectively by a statistical method, which showed that the data fit equations of the three halves order much better than those of either first or second order reactions. The reason for this unexpected behavior has not yet been established. In any case, the equation of a three halves order reaction affords a reasonably accurate basis for the quantitative description of the process of destruction of hemagglutinin and for computing the specific reaction velocity. The specific reaction velocity varies in an inverse manner with the initial virus concentration.

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# Precipitation and Recovery of Malt and Mold Amylase by Alkali Cook Lignin

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Received August 14, 1945

## INTRODUCTION

In a previous communication (1), the authors described a method for the recovery of enzymes from aqueous solution by precipitation in mildly acid solution with certain forms of lignin and tannin.

The process was described in relation to a mold protease derived from *A. flavus* Link. In the current investigation, the method was successfully applied to the recovery of malt and  $\beta$ -diastase.

A study of the reconstituted malt diastase precipitate was of interest not merely because it represented a diastatic instead of a proteolytic preparation, but also because its optimum enzymatic activity occurred on the acid side (pH 5.5), not far from the pH at which it had been precipitated. In the previous study, enzymatic activity was tested in all cases at pH 7.5, which corresponded both with complete re-solubilization of the acid-precipitated enzyme-lignin and with the optimum activity of the tryptic mold protease.

The malt enzyme proved to be more susceptible to inactivation than the previously studied mold protease, particularly on exposure to alkali. Various factors, including the absolute concentration of protein in the solution as well as the relative concentration of protein to lignin, significantly influenced the extent of precipitation and reconstitution. By comparing the activity of the enzyme-lignin at various hydrogen ion concentrations with that of the original enzyme infusion, it was found that the reactivation of the precipitated diastase-lignin complex was a function of the hydrogen ion concentration.

The lignin method was also successfully applied to the recovery of mold  $\beta$ -diastase derived from *A. oryzae*. In general, the fungal diastase resembled the malt diastase in its behavior, being, however, somewhat more acid-stable. Optimum recovery of the mold enzyme was achieved

when the precipitation was carried out at approximately pH 3.5, whereas optimum precipitation of the malt diastase took place at approximately pH 4.5.

The addition of gelatin and of excess lignin under certain conditions widened the pH range in which optimum recovery could be achieved, and helped protect both enzymes against inactivation by acid.

#### MATERIALS AND METHODS

The lignin employed was a 5% solution of alkali-cook lignin (2) dissolved in *N*/10 sodium hydroxide. Because of the alkali sensitivity of the diastases, it was necessary to remove most of the alkalinity required to dissolve the lignin. This was done by a 48 hour dialysis against running tap water to a pH of 8.4. Final adjustment to volume was made with distilled water. Careful neutralization of the dissolved lignin with very dilute mineral acids could also be employed.

The malt diastase infusion was prepared and assayed by the  $\beta$ -amylase method of the American Society of Brewing Chemists (3); this method, based on a Fehling titration of the digested soluble starch using methylene blue as an indicator, was also employed for determination of the Lintner values of the reconstituted precipitates and the infusions of mycelial diastase.

The fungal enzyme was derived from the growth of *A. oryzae* on moistened bran. The infusion was prepared by agitating a given weight of mold bran with 20 times its weight in water on a shaking machine for one hour, followed by Buchner filtration. Both diastase solutions were stable for at least four hours at room temperature, and throughout the day if kept on ice.

Fifty cc. portions of the mycelial and malt diastase extracts were employed for the precipitations. After addition of the lignin to the malt infusion, acidification was carried out by means of 1% phosphoric acid which was added drop-wise with vigorous agitation to the requisite pH. For the mold diastase, where higher acidities were required for precipitation, a 5% phosphoric acid solution was employed.

The acidified solutions were centrifuged for 15 minutes at 2500 r.p.m. Drying of the centrifuged enzyme-lignin precipitate was carried out in the presence of a small quantity of mixed primary and secondary sodium phosphate so as to yield a final pH of approximately 6.5.

During the drying, the centrifuged neutralized precipitate was spread on a flat surface in a current of air from an electric fan. Under these conditions no loss of activity occurred on drying. The dried material was stable for several months at room temperature.

Prior to assay, the dried precipitate was redissolved directly in water to the original volume of the infusion.

For assay of the respective enzyme fractions, except as otherwise specified, digestion of the starch was carried out in *M*/50 phosphate buffer at pH 6.5. Where gelatin was added to aid the precipitation, the order of addition was lignin, then gelatin, then phosphoric acid.

Nitrogen determinations were made by semi-micro Kjeldahl and by the Nessler reagent. Hydrogen ion concentrations were determined by the Beckman pH meter.

## EXPERIMENTAL

The precipitation of malt diastase by lignin was investigated as to quantity. Varying amounts of 5% solution of alkali cook lignin were added to 50 cc. of a 5% malt diastase infusion of 150° Lintner containing 0.23 mg. nitrogen per cc. The solutions were acidified to pH 4.6 by the dropwise addition of 1% phosphoric acid, the total volumes adjusted to 55 cc., the mixtures centrifuged and the precipitates dried as described above. The dried precipitates were reconstituted to their original volume (50 cc.) and tested for  $\beta$ -amylase after digestion at pH 6.5.

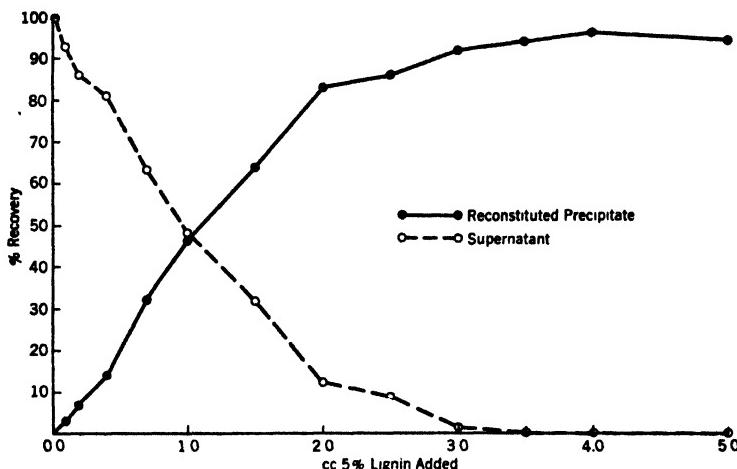


FIG. 1  
Precipitation of Malt  $\beta$ -Diastase by Varying Concentrations  
of Alkali-Cook Lignin at pH 4.6

Comparative recoveries in terms of the original solution as well as the amount of enzyme remaining in the supernatant are shown in Fig. 1. It will be seen that the amount of enzyme precipitated under these conditions is a function of the concentration of lignin added.

The extent of enzyme recovery was investigated in regard to the hydrogen ion concentration at which the precipitation occurred. Using 3 cc. of the lignin solution, a series of precipitations was carried out on an infusion of 163° Lintner and 0.24 mg. nitrogen per cc. at various hydrogen ion concentrations ranging from pH 3.3 to 5.8.

The respective amounts of enzyme recovered in the precipitate and those remaining in the supernatants are shown in Fig. 2. It will be seen that maximum recovery is achieved in the vicinity of pH 4.5. Increasing

the acidity beyond this point results in steady inactivation of the  $\beta$ -amylase. Decreasing acidities cause incomplete precipitation, the unprecipitated enzyme remaining in the supernatant. A broadening of the area of maximum recovery may be achieved by increasing the amount of lignin added to 5 cc. (see broken line).

A study was made of the influence of protein and enzyme concentration on the precipitation of malt diastase at varying hydrogen ion concentrations, ranging from pH 3.4 to 5.4.

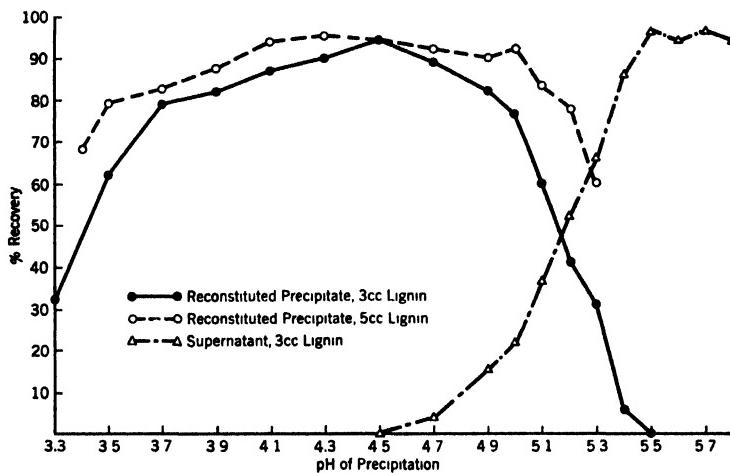


FIG. 2  
Recovery of Malt Diastase by Alkali-Cook Lignin at Varying Hydrogen Ion Concentrations

In this experiment, shown in Fig. 3, a 5% malt infusion of 146° Lintner and containing 0.22 mg. nitrogen per cc. was compared with a 2.5% infusion of 78° Lintner containing 0.12 mg. nitrogen per cc. and a 10% infusion of 239° Lintner containing 0.41 mg. nitrogen per cc. The samples were prepared by shaking the ground malt with distilled water in a mechanical shaker for one hour at room temperature, and then filtering through a Buchner funnel. Precipitation was carried out with 3 cc. of the 5% lignin solution.

It will be seen that greater recovery values are achieved in the more concentrated infusions. The differences, however, are not substantial.

In a further experiment, the 2.5% malt infusion was supplemented by the addition of 0.2 cc. of 2% gelatin to 50 cc. of the infusion, after the

lignin addition, but prior to acidification. It will be seen (from the broken line in Fig. 3) that, by the gelatin addition, recovery of the enzyme is aided in the upper and lower segments of the curve. The nitrogen content of the infusion was increased by the gelatin addition only from 0.12 to 0.14 mg. per cc. indicating that it is the qualitative nature of the supplemental protein which is of importance.

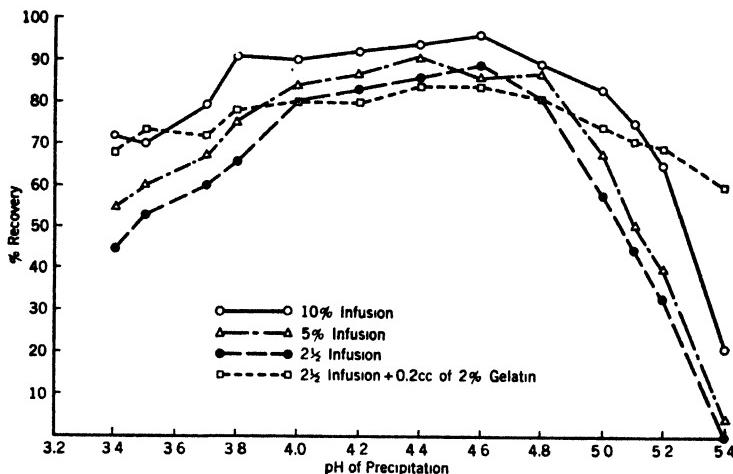


FIG. 3  
Influence of Nitrogen and Enzyme Concentration on Recovery  
of Malt Diastase by Lignin

None of the supernatants resulting from precipitations of the 2.5% infusion below pH 4.0 showed any appreciable activity. The greater recovery resulting from gelatin addition in the lower hydrogen ion concentrations thus represents a protection of the enzyme against inactivation.

Also, in more concentrated diastatic infusions, by the addition of gelatin, a substantial broadening of the range of maximum recovery in the direction of neutrality could be achieved.

Fig. 4 shows the precipitation of 50 cc. of a 5% malt infusion of 165° Lintner and 0.24 mg. nitrogen per cc. with 4 cc. lignin in the presence, and in the absence, of 0.2 cc. of 2% gelatin.

It will be seen that the addition of gelatin broadens the upper pH limit at which at least 90% recovery was achieved from 4.9 to 5.4.

The preceding diastase digestions were carried out in  $M/50$  phosphate buffer at pH 6.5. This hydrogen ion concentration was selected to insure complete solubility of the precipitated enzyme-lignin, and thus full activity of the reconstituted enzyme. The optimum activity of malt diastase, however, is at a somewhat lower hydrogen ion concentration. It was of interest, therefore, to compare the activity of the protein-lignin complex with that of the original infusion at various ranges throughout the pH curve.

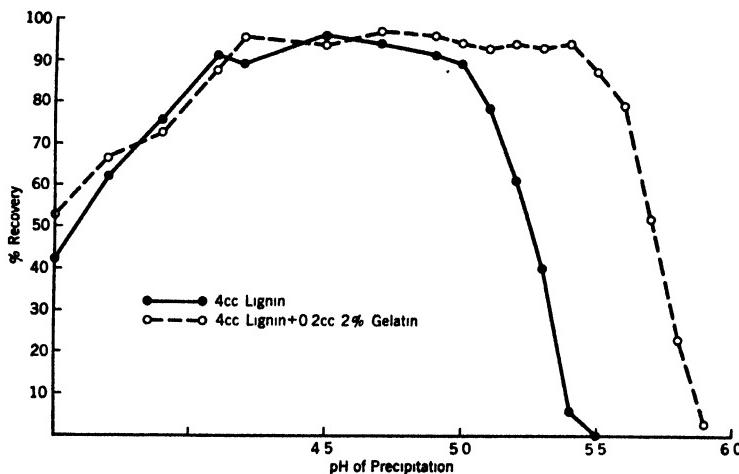


FIG. 4

Influence of Gelatin Addition on the Recovery of Malt Diastase by Lignin

For this comparison, the diastase lignin was precipitated at pH 4.4 with 3 cc. of 5% lignin. The precipitates thus prepared were suspended in buffers of mixed primary and secondary phosphate which had been adjusted to various pH's ranging from 4.4 to 8.8. The same respective buffers at  $M/50$  concentration were employed in each case for determination of the Lintner values. The Lintner activity of the original infusions was likewise determined in the respective buffers.

Comparison of the activity of the reconstituted enzymes and the original infusions at their respective hydrogen ion concentrations is illustrated in Fig. 5.

Maximum activity of the enzyme-lignin complex is not achieved until pH 6.5 at which better than 90% recovery is achieved in relation to

pH 6.5 tested infusion. At higher hydrogen ion concentrations, proportional recovery is virtually complete but the extent of diastatic activity falls off substantially. At hydrogen ion concentrations below pH 6.5, the activities of the lignin-precipitated diastase preparations fall off markedly, both absolutely, and in relation to their respective infusions.

Application of the lignin recovery to another diastase, a mold amylase derived from *A. oryzae* was studied in respect to the conditions for optimum precipitation. This enzyme was precipitated, dried and reconstituted as previously described.

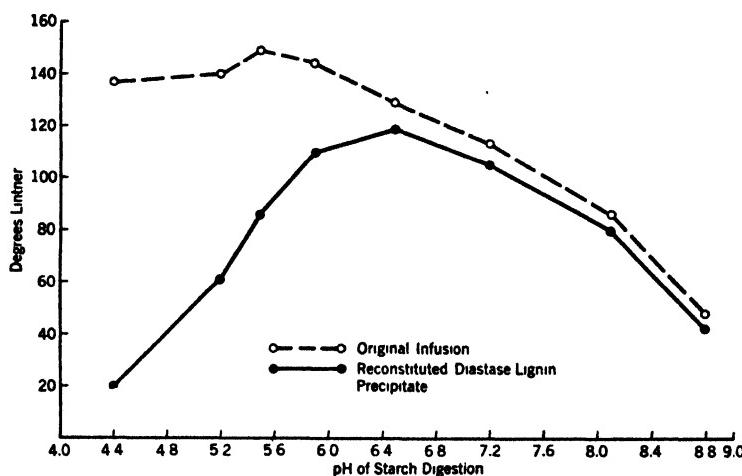


FIG. 5  
Effect of pH on the Apparent Dissociation and Activity  
of the Malt Diastase-Lignin Complex

Activity of the fungal diastase was determined by digestion in the presence of *M/50* phosphate buffer at pH 6.5.

Fig. 6 illustrates the precipitation of a 5% extract of the mycelial enzyme containing 0.66 mg. of nitrogen per cc. by 5 cc. of the 5% alkali cook lignin solution at various hydrogen ion concentrations ranging from 2.5 to 5.0. The degree of recovery is illustrated in contrast to a Lintner value of 161° found in the infusion. It will be seen that the mold amylase is more acid-stable than the malt diastase under corresponding conditions (see Fig. 2) and its recovery optimum lower in respect to pH.

The addition of 0.5 cc. and 1.0 cc. of 2% gelatin to the mixture prior to precipitation considerably broadened the pH range in which maximum recovery of the fungal diastase could be achieved. The addition of excess gelatin (2 cc. of the 2% solution), however, was found to interfere with the precipitation and yielded lower recovery values than were achieved without gelatin addition.

The comparative diastatic activity of the mycelial extracts and their reconstituted lignin precipitates was determined at varying hydrogen ion concentrations ranging from pH 4.0 to pH 9.2. Digestions of the

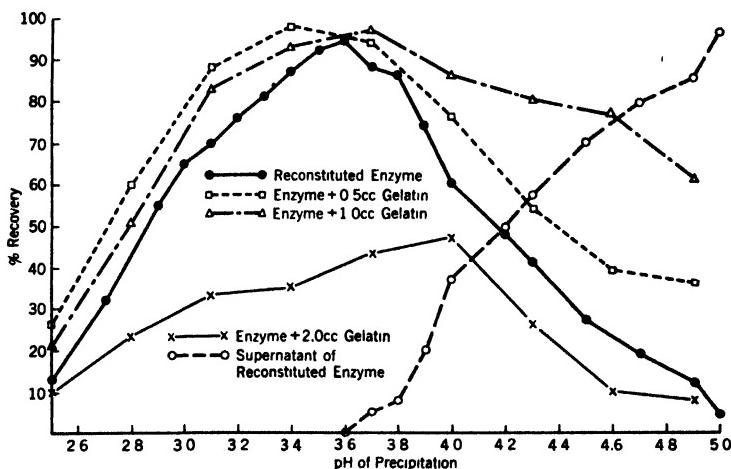


FIG. 6  
Recovery of Mold Diastase Precipitated at Various Hydrogen Ion Concentrations in the Presence and Absence of Gelatin

reconstituted mold diastase precipitated at pH 3.6 with 5 cc. lignin solution were carried out at the respective hydrogen ion concentrations in mixed phosphate buffers following the method previously employed with malt diastase (see Fig. 5). Activity of the respective original infusions and the reconstituted enzyme are illustrated in Fig. 7. It will be seen that the mold diastase-lignin complex becomes solubilized and reactivated at a somewhat greater hydrogen ion concentration than the previously investigated malt diastase complex. The fungal enzyme is in turn somewhat more alkali-sensitive than the malt diastase, this sensitivity being accentuated in the lignin precipitate.

## DISCUSSION

In our previous study of the precipitation of protease from *A. flavus* Link by means of alkali-cook lignin it was found that this method was well suited to the recovery of an enzyme having its maximum activity at a somewhat alkaline hydrogen ion concentration. The current study indicates that the lignin precipitation method may also be successfully applied to enzymes whose optimum activity occurs at mildly acid hydrogen ion concentrations.

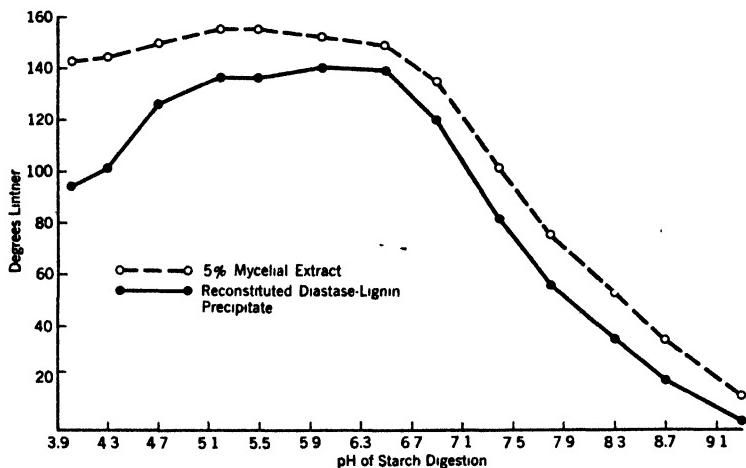


FIG. 7  
Comparative Diastatic Activity at Varying Hydrogen Ion Concentrations of  
Mycelial Extracts and Their Reconstituted Lignin Precipitates

Complete reconstitution of the precipitated enzyme at its range of maximum activity is dependent on the degree of dissociation or resolubilization of the enzyme-lignin complex at this point, a factor which varies with the enzyme.

Factors of protein and enzyme concentration, lignin concentration, ratio of protein to lignin, the acid or alkali sensitivity of the enzyme at any stage of the precipitation process and the influence of protective colloids are likely to vary in their influence from one enzyme to another and must thus be studied individually before application of the lignin precipitation method to any given enzyme can be successful.

In our investigation on the precipitation of mold protease, successful recoveries were achieved with tannic acid and certain other forms of

tannin. We were unable, however, to duplicate these findings with respect to malt or fungal diastase, and always encountered a substantial amount of inactivation in the course of the tannic precipitation.

### SUMMARY

1. The lignin process for the precipitation and recovery of enzymes may be successfully applied to malt  $\beta$ -amylase. Almost complete recovery is achieved in terms of the original enzyme when the determination is carried out at a pH at which the enzyme lignin is fully dissociated and thus completely reactivated (pH 6.5 or higher). At the pH of optimal activity of the malt diastase, however, (pH 5.5), the comparison is somewhat less favorable due apparently to incomplete solubility of the enzyme-lignin.

2. Optimum recovery of malt  $\beta$ -amylase occurs when the precipitation is carried out at pH 4.5. Below this point gradually increasing inactivation is likely to occur. At higher pH values precipitation is likely to be incomplete, the unprecipitated enzyme remaining in the supernatant.

3. A mold  $\beta$ -amylase derived from *A. oryzae* was also successfully recovered by the lignin method. This enzyme, somewhat more acid-stable than the malt diastase, had its optimum precipitation range in the vicinity of pH 3.6. Resolubilization and reactivation of the fungal  $\beta$ -diastase also took place at a lower pH range, exemplifying the individual differences in the reaction of enzymes to the lignin precipitation.

4. Drying of the two lignin-precipitated diastase preparations could be carried out without loss in the presence of a mixed phosphate salt at approximately pH 6.5. The enzymes were alkali sensitive, and became inactivated when exposed to higher hydrogen ion concentrations. In dried form, the enzyme preparations were stable for several months at room temperature.

5. The addition of gelatin under certain conditions was found to protect both the malt and the mold diastase against inactivation. Gelatin was also found to broaden, in the direction of neutrality, the pH range at which maximum recovery could be obtained. The addition of excess gelatin, however, will interfere with the precipitation.

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# A Simple Method for the Determination of Glucose in Blood

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Received August 7, 1945

## INTRODUCTION

The determination of glucose in blood is more frequently made in clinical laboratories than any other chemical procedure. For routine purposes there is a choice between the colorimetric methods of Folin and Wu (1) and of Folin and Malmros (2). In the Folin-Malmros procedure potassium ferricyanide is reduced by glucose in carbonate-cyanide solution and the amount of ferrocyanide formed is estimated by the blue color obtained on addition of a ferric salt.

The ferricyanide reduction method has two main advantages over the earlier procedures based on the reduction of copper salts: (a) under suitable conditions, the amount of ferrocyanide obtained is accurately proportional to the amount of glucose used (3) and (b) ferrocyanide is not readily reoxidized on exposure to air so that regular test tubes can be used in this procedure instead of the special tubes with a constriction, required for the Folin-Wu method.

Both methods were originally designed for the use of visual colorimeters. When photoelectric instruments became standard equipment in most clinical laboratories, the Folin-Wu and Folin-Malmros procedures were adapted for readings in such colorimeters without any noteworthy change.

Horvath and Knehr (4) studied the various factors responsible for the variability of glucose recovery in standard solutions with the Folin-Malmros method. The influence of heating time on color density was reinvestigated, and data concerning color stability and speed of color development under various conditions were collected. The method finally adopted by Horvath and Knehr incorporated a number of suggestions by earlier workers, such as the replacement of gum ghatti by a synthetic emulsifying agent. A calibration curve which was not a straight line on semi-logarithmic paper was obtained. In order to permit use of this curve, it was necessary to include a standard solution (100 mg. %) of glucose in each test series and to

adjust the photoelectric instrument so that the standard would read 60% transmission. Through this arrangement the authors were able to compensate for the continuous increase in density of the unknown solutions. As a result of these modifications it became possible to recover glucose between 50 and 300 mg. % with a maximal error of 3.5%.

Despite the improvements made by Horvath and Knehr the Folin-Malmros procedure is not ideal for the routine clinical laboratory. It occurred to us that the determination of glucose in blood would be considerably simplified, if it were possible to measure glucose directly by a photoelectric determination of the disappearance of ferricyanide. A procedure based on this idea had already been suggested in 1937 by Hoffman (5). There are, however, a number of reasons why his method could not find universal acceptance:

- (a) special tubes with a mark at 8 ml. were required,
- (b) as the calibration curve was not a straight line, the ferricyanide reagent had to be prepared with great accuracy and could no longer be used when inevitable, slight changes in concentration became noticeable, and
- (c) the curve covered only blood glucose values up to 200 mg. % and repetition of the determination with a smaller aliquot was, therefore, necessary when higher concentrations were encountered.

In view of the extreme simplicity and speed offered by the principle on which this method is based, it was decided to reinvestigate its practical possibilities. This has resulted in a simple and accurate routine method in which the objections against the earlier procedure (5) have been completely eliminated.

In the new method, regular test tubes with a mark at 25 ml. are used. The concentration of the ferricyanide reagent can be varied within limits of at least 20% and the straight line calibration curve covers the range up to 500 mg. % of blood glucose. The development of the new method was made possible by the availability of a sensitive photoelectric colorimeter in combination with a suitable narrow band filter and by the use of a buffered ferricyanide solution as reagent.

## EXPERIMENTAL

*Instrument.* A Lumetron photoelectric colorimeter No. 402-E in combination with filter M-420 was used in the present investigation. Regular Pyrex test tubes with an

outside diameter of 18 mm. were found suitable as colorimeter tubes. Tubes were selected which, when inserted into the test tube adapter in a marked position, gave under actual testing conditions with phenol red solutions and filter M-540 practically identical readings with a maximal deviation of not more than  $\pm 0.44\%$  from the average extinction value. It was found that about 28–30 tubes out of 100 fell in this range of uniformity. With this equipment, potassium ferricyanide solutions followed Lambert-Beer's law closely (Fig. 1) as was to be expected from Kortüm's (6) results with monochromatic light.

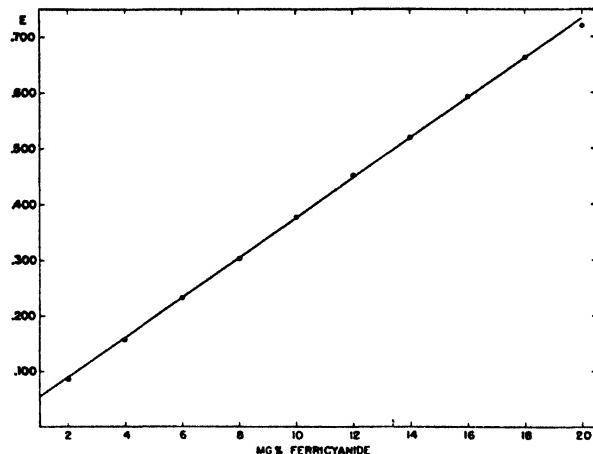


FIG. 1

#### Relationship Between Concentration and Extinction of Potassium Ferricyanide Solutions

*Alkalinity of Ferricyanide Solution.* The reduction of ferricyanide by glucose takes place in alkaline solution. Folin and Malmros (2) used a mixture of sodium carbonate and sodium cyanide to achieve the required alkalinity and this mixture was also used by Horvath and Knehr (4). Klendshoj and Hubbard (7) reported that satisfactory results could be obtained with carbonate alone, but Horvath and Knehr noted, in the absence of cyanide, non-proportional color of the unknown solution in relation to the standard for about 25 min., after which the readings remained relatively stable.

The use of a strongly poisonous cyanide solution for daily routine testing seems undesirable. Furthermore, the presence of cyanide more than doubles the amount of ferricyanide reduced by a given quantity of glucose. Although this is of advantage for visual colorimetric work, it limits considerably the range of glucose concentrations which can be read in a photoelectric instrument. If sodium carbonate alone is used, its concentration has to be kept constant. A significant difference in the reducing power of 0.1 mg. glucose was noted when the sodium carbonate concentration in the mixture during heating was 0.33 instead of 0.30%. About 13% less ferricyanide

was reduced when the reaction mixture contained 0.40% instead of 0.30% sodium carbonate.

Our observations are in accord with findings by Fujita and Okamoto (8), who noted considerable effects of small changes in pH on the reducing power of glucose towards ferricyanide. The source of error due to slight changes in alkalinity was eliminated by the use of a phosphate buffer instead of a carbonate solution.

*Reagent.* 1. STOCK SOLUTION. Dissolve 2.5-3.0 g. potassium ferricyanide, 140 g. anhydrous di-potassium phosphate and 42 g. tertiary potassium phosphate in 1000 ml. of water. Store in a brown bottle in the dark. (This solution remained clear and showed no significant change in optical density over a period of 4 months.)

2. DILUTE REAGENT. Dilute daily sufficient stock solution about 1:5 with distilled water to last for one day's work.

*Procedure.* Into test tubes with a mark at 25 ml., pipette 1 ml. Folin-Wu filtrate and add exactly 10 ml. dilute reagent. Mix by lateral shaking. Prepare a blank tube with 1 ml. of water instead of filtrate and add also 10 ml. dilute reagent. Keep the tubes in boiling water for 15 min. and cool afterwards for about 3 min. by immersion in cold water. Fill up to the 25 ml. mark with distilled water and mix. Transfer portions to colorimeter tubes and read in a sensitive photoelectric colorimeter against distilled water, using a narrow band filter with a maximum transmission at about 420 m $\mu$ . The blank tube should have a transmission value of 15 to 25%.

*Calculation.* The extinction (or density)  $E$  of each solution is calculated from the per cent transmission values ( $I$ ) of each sample:

$$E = \log \frac{I_0}{I}.$$

As the instrument is usually adjusted so that distilled water reads 100% transmission, the equation becomes:

$$E = \frac{\log 100}{\log I} = 2 - \log I.$$

The extinction of the unknown ( $E_u$ ) is subtracted from the extinction of the blank ( $E_b$ ) and this decrease in extinction,  $\Delta E = E_b - E_u$ , was found to be proportional to the glucose concentration of the unknown ( $C_u$ ):

$$C_u = (E_b - E_u) \times F.$$

The value of the calibration factor  $F$  depends not only on the procedure but also on the type of instrument and filter used.\*

*Calibration Factor.* Ten glucose standard solutions were prepared from anhydrous glucose which corresponded in glucose concentration to filtrates from blood containing 50–500 mg. % glucose. One ml. of each standard solution was subjected to the test; the resulting calibration curve is shown in Fig. 2. (All data in Fig. 1 and 2 and in the

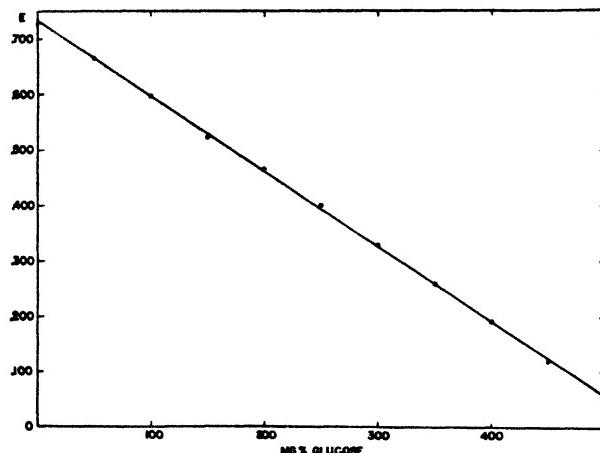


FIG. 2

Calibration Curve, Showing the Decrease in Extinction of a Phosphate Buffered Ferricyanide Solution After Heating with Various Amounts of Glucose

Tables in this report are derived from single determinations and are not average values from duplicate or triplicate tests.)

The decrease in extinction per 100 mg. % glucose was calculated for each reading shown in Fig. 2 and amounted to 0.131 with a maximal deviation of 3%. The value of the calibration factor was thus found to be 100/0.131 or 763. The extinction of a 10 mg. % potassium

\* The extinction value for a given transmission can be looked up in charts. If such a chart is not available, a logarithmic table allows the following short cut calculation of  $(E_b - E_u)$ :

$$E_b = 2 - \log I_b; \quad E_u = 2 - \log I_u.$$

Therefore,  $(E_b - E_u) = (2 - \log I_b) - (2 - \log I_u) = \log I_u - \log I_b$ . Example: if the blank transmits 20% light and the unknown 50%,  $(E_b - E_u) = \log 50 - \log 20 = 1.699 - 1.301 = 0.398$ .

ferricyanide solution was  $0.379 \pm 0.005$  (Fig. 1). A decrease in extinction by 0.131 corresponds, therefore, to the reduction of 0.864 mg. ferricyanide in 25 ml. solution, so that under the conditions of our test one molecule of glucose reduces 4.7 molecules of ferricyanide.

*Recovery Experiments.* One ml. each of various glucose standard solutions was added to a number of tubes containing 1 ml. Folin-Wu filtrate. The results of the glucose determinations in these mixtures, listed in Table I, show that known amounts of glucose were recovered with a maximal error of 2.7%.

TABLE I  
*Recovery of Known Amounts of Glucose added to a Folin-Wu Filtrate*

Added mg. % glucose	Found mg. % glucose	Recovered mg. % glucose	Error in per cent of amount added
0	110	—	—
100	209	99	-1
150	260	150	0
200	312	202	+1
250	364	254	+1.6
300	418	308	+2.7

*Effect of Heating Time and of Standing.* In the Folin-Malmros procedure the heating time was found to have a considerable influence on the results (4). In the method described here, the reaction of glucose with ferricyanide was practically complete after 5 min. and little change was observed on prolonged heating as shown in Table II. Accurate timing of the period the tubes are left in boiling water is, therefore, not necessary. For a comparison, data estimated from Fig. 1 of the paper by Horvath and Knehr (4) are also listed in Table II.

TABLE II  
*Effect of Heating Time on Light Transmission*

Heating Time min.	Transmission per cent			
	Schales and 150 mg. %	Schales 300 mg. %	Horvath and 150 mg. %	Knehr (4) 300 mg. %
5	32.8	51.7	64	40
10	32.6	52.1	52	31
15	33.0	52.3	47	27

Standing of the tubes for 2 hrs. at room temperature after they had been filled up to the 25 ml. mark, had no effect on light transmission.

It is, therefore, not necessary to read the tubes at any definite interval after completion of the test.

*Determination of "True" Glucose in Blood.* A Folin-Wu filtrate contains, in addition to glucose, other reducing materials which are responsible for about 20–30 mg. % of the so-called normal glucose values in human blood. Most successful among the various procedures recommended to eliminate these non-fermentable substances together with the precipitation of proteins is the cadmium hydroxide method of Fujita and Iwatake (9). In the method described here, "true" glucose values are obtained simply by substituting 1 ml. cadmium hydroxide filtrate for 1 ml. Folin-Wu filtrate. A comparison of results obtained with both types of filtrates is shown in Table III. The presence of small amounts of cadmium in the filtrate does not interfere with our method so that their removal with barium carbonate (3) is not necessary.

TABLE III

*Comparison of Blood Glucose Values obtained with Folin-Wu and Cadmium Hydroxide Filtrates*

No.	Mg. per cent glucose Folin-Wu	Mg. per cent non-glucose $\text{Cd}(\text{OH})_2$ (as glucose equivalent)
9	125	95
10	102	78
24	189	160
26—F	109	80
26—1 hr.	157	129
26—2 hrs.	102	73
81—F	110	83
81—½ hr.	159	136
81—1 hr.	184	161
81—2 hrs.	124	101
81—3 hrs.	131	108

(Samples No. 26 and 81 were taken before and at the specified intervals after the oral administration of 100 g. glucose flavored with lemon juice.)

#### SUMMARY

1. A method of determining the concentration of glucose in blood by photoelectric measurement of the disappearance of ferricyanide from a phosphate buffered solution is described.
2. The method described here covers the range up to 500 mg. % glucose and can be used for Folin-Wu filtrates as well as for cadmium

hydroxide filtrates. The maximal error in recovery experiments was below 3%.

3. The decrease in extinction of a ferricyanide solution on heating with a glucose solution is proportional to the amount of glucose present, provided that a sensitive photoelectric instrument with a narrow band filter (maximum transmission 420 m $\mu$ ) is used.

4. Small changes in the concentration of the reagent, variation of the heating time from 5 to 15 minutes or standing of the solutions for 2 hours before readings were taken, had little or no effect on the results.

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## **Studies on the Biochemistry of *Tetrahymena***

### **V. The Chemical Nature of Factors I and III**

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Received August 27, 1945

#### **INTRODUCTION**

It was reported earlier (1, 2) that the ciliate *Tetrahymena geleii* W required two growth factors of unknown composition in addition to known vitamins, inorganic salts and certain specific amino acids (3). These unknown growth factors were designated Factors I and II and were found regularly in many materials of plant and animal origin (timothy hay, crude casein, egg yolk, peptones, etc.). They could be separated by precipitation with the salts of heavy metals (most satisfactorily by lead acetate). Factor I was precipitated out and Factor II remained in the filtrate. Later, another unknown growth factor for *Tetrahymena*, found to be present in proteins and protein hydrolysates as well as in some crude plant preparations, was also described (4). This was designated Factor III. It was shown that growth failed in amino acid media supplemented with Factors I and II, but was successful in protein hydrolysates. Factor III was found to be stable to strong acids while Factors I and II were unstable. Tests have shown that no one of these three factors could be replaced by thiamine, riboflavin, pantothen, biotin, nicotinic acid or its amide, pyridoxine, folic acid, choline or inositol, either singly or in combinations.

A continuation of the work on the isolation of the unknown factors for *Tetrahymena* has been going on and some of the properties suggested that nucleic acid or derivatives of nucleic acid might be involved. Accordingly tests were made with that possibility in mind and the results of those experiments have enabled us to remove Factors I and III from the "unknown" list. Factor II remains unknown as yet.

<sup>1</sup> Aided by grants from the Morgan Edwards Fellowship Fund and the Manufacturers Research Fund for Bacteriology and Protozoology of Brown University.

### EXPERIMENTAL

The organism used in the work to be reported was *Tetrahymena gelei* W, grown in pure (bacteria-free) culture. Biochemical and serological descriptions of this strain are to be found elsewhere (5, 6, 7, 8).

Two types of base media were employed. One consisted of a 0.5% acid hydrolysate of Eastman purified calfskin gelatin (lot no. 144) prepared in the manner previously described (7). To the hydrolysate was added 0.1 mg. % *l*(*-*)-tryptophane and 0.2 mg. % *dl*-valine.

The second base medium was a mixture of the eleven amino acids found to be essential for optimum growth of this strain of ciliate (3). The concentrations of each of the amino acids has been given previously (7).

Both of the above base media were supplemented with the following:

	mg./ml.
Difco bacto dextrose . . . . .	2.00
MgSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0.10
K <sub>2</sub> HPO <sub>4</sub> . . . . .	0.10
CaCl <sub>2</sub> ·2H <sub>2</sub> O . . . . .	0.05
FeCl <sub>3</sub> ·6H <sub>2</sub> O . . . . .	0.00125
MnCl <sub>2</sub> ·4H <sub>2</sub> O . . . . .	0.00005
ZnCl <sub>2</sub> . . . . .	0.00005
 γ/ml.	
Biotin methyl ester . . . . .	0.00005
Calcium pantothenate . . . . .	0.10
Thiamine hydrochloride . . . . .	0.10
Nicotinamide . . . . .	0.10
Riboflavin . . . . .	0.10
Pyridoxine hydrochloride . . . . .	0.10
<i>p</i> -Aminobenzoic acid . . . . .	0.10
Folic acid <sup>2</sup> . . . . .	0.10
<i>t</i> -Inositol . . . . .	1.00
Choline chloride . . . . .	1.00

The sources of the above substances have been given previously (3).

The complete gelatin hydrolysate base medium will be designated EGH and the complete amino acid-base medium 11AA.

The Factor II used in this work was prepared in the following manner. Liver Fraction L<sup>1</sup> (50 g.) was dissolved in 1 l. of distilled water and to this was added a 40% solution of normal lead acetate until no further precipitation occurred. The precipitate was removed by filtration with the aid of Celite. The filtrate was neutralized with NaOH and treated with an excess of basic lead acetate. After removal of the basic lead acetate precipitate, the filtrate was treated with oxalic acid to remove

<sup>2</sup> Folic acid concentrate with a "potency" of 5,000, furnished through the courtesy of Dr. R. J. Williams.

<sup>1</sup> Furnished through the courtesy of Dr. David Klein and the Wilson Laboratories.

the excess lead, filtered and then treated with  $\text{Ca}(\text{OH})_2$  to remove the oxalic acid. This treatment was stopped when the solution reached neutrality and the oxalate was filtered off. This filtrate was found to contain some Factor I and Factor III activity but these traces could be almost completely removed by treating with activated charcoal. The filtrate was adjusted to pH 3.5 and 10 g. of Norit A was added. After stirring for one hour at room temperature the Norit was filtered off and discarded and the filtrate was neutralized. The amount of Norit added and the temperature at which adsorption takes place are important, for it was found that while Factor II is less readily adsorbed than Factors I and III, the Factor II activity is reduced after adsorption at increased temperatures and may be completely adsorbed if enough Norit is used. This final filtrate, to be referred to hereafter as Factor II, was stored at 2°C. under toluene. Factor II was used in concentrations of 1 part in 10 parts of final medium.

Many other techniques were employed for the preparation of Factor II but none were as satisfactory as the above. The use of copper and silver salts as precipitants gave preparations containing appreciable amounts of Factors I and III, while the use of timothy, alfalfa and Cerophyl extracts as starting materials produced preparations much lower in Factor II activity.

The organisms for all of the experimental series were grown in  $125 \times 7$  mm. Pyrex test tubes in 2 ml. volumes of medium. Transplants were made at 72 hour intervals with bacteriological loops delivering approximately 0.005 ml. of fluid. At least three serial transplants were made in all cases to effectively reduce the carry-over of chemicals. The population densities were determined by the direct counting technique (9) and the results recorded represent those obtained in the third serial transplant after 72 hours of incubation at 25°C.

The sources of the nucleic acid and nucleic acid derivatives used were as follows: yeast nucleic acid, guanine hydrochloride, adenine sulfate, xanthine and uracil purchased from Eastman Kodak Co.; cytosine, cytidine, cytidylic acid, thymine and uridylic acid received as a gift from the Levene Collection through the courtesy of Mr. Edric B. Smith of the Rockefeller Institute; adenylic acid (from yeast), guanosine and hypoxanthine received as a gift from Dr. J. P. Greenstein of the National Institute of Health. All of these substances, including the yeast nucleic acid, were found to be pure to the extent that they were biuret and ninhydrin negative. They were all free of riboflavin, pantothen, biotin, nicotinic acid or its amide, and pyridoxine, as determined by the *Lactobacillus casei* test, and all, except the yeast nucleic acid, were free of folic acid.

In addition to the above, liver nucleic acid was prepared from fresh beef liver by the method outlined by Levene (10).

The nucleic acids and all of the nucleic acid derivatives were used in concentrations of 0.05 mg. per ml. of final medium.

## RESULTS

By employing two types of base media, one (EGH) containing Factor III and the other (11AA) containing no growth factors, it was possible to test a wide variety of substances for growth factor activity.

The additions of Factor II alone failed to support growth beyond the first transplant in EGH while barely transplantable growth occurred in 11AA. This means that Factor II preparations are contaminated with traces of Factors I and III, but that inhibitory substances are present in the gelatin hydrolysate which cannot be overcome by these traces. The growth which occurs in 11AA is so low, however, that the combination is satisfactory for all but precise quantitative work on the unknown factors. Additions of yeast nucleic acid or liver nucleic acid, either untreated or hydrolyzed with ammonia water, failed to support growth beyond the first transplant in either base medium (Table I).

TABLE I

Supplements	Base media	
	EGH <sup>4</sup>	11AA <sup>4</sup>
Factor II	0	150
Yeast nucleic acid (YNA)	0	0
Liver nucleic acid (LNA)	0	0
Hydrolyzed yeast nucleic acid (HYNA)	0	0
Hydrolyzed liver nucleic acid (HLNA)	0	0
Factor II + YNA	34,000	52,000
Factor II + LNA	38,000	51,000
Factor II + HYNA	181,000	240,000
Factor II + HLNA	160,000	210,000
Factor II + adenine	0	0
Factor II + guanine	80,000	110
Factor II + xanthine	24,000	140
Factor II + hypoxanthine	0	0
Factor II + adenylic acid	17,000	90
Factor II + guanosine	78,000	120
Factor II + cytosine	0	110
Factor II + cytidine	0	160
Factor II + cytidylic acid	0	100
Factor II + uracil	0	150
Factor II + uridylic acid	0	80
Factor II + thymine	0	200

Nucleic acid (either yeast or liver) together with Factor II resulted in the production of indefinitely transplantable growth to fair concentrations in both types of base media. This means that some components of the nucleic acid molecule can substitute for Factors I and III. The rate of growth was low, however, and the concentrations never reached

<sup>4</sup> The figures represent the number of ciliates per ml. in the third serial transplant after 72 hours growth.

optimum until after seven or eight days. When the nucleic acids were hydrolyzed the growth rate increased as did the maximum yield. This indicates that the ciliates are able to break down the nucleic acid molecule but that speed of growth is increased if hydrolysis is performed for them.

Tests with nucleic acid derivatives were set up to determine the specific components which were responsible for Factors I and III activity. We were able to obtain a limited number of nucleotides, nucleosides and bases, and these were tested in all combinations. When each of the components was added singly (together with Factor II) to each of the base media, it immediately became apparent that the Factor I activity resided in the purines and the Factor III activity in the pyrimidines (Table I). Thus, none of the purines (guanine, adenine, xanthine, hypoxanthine, guanosine, adenylic acid) had any stimulatory effect in 11AA, while all but adenine and hypoxanthine produced moderate to fair growth in EGH. On the other hand the pyrimidines (cytosine, cytidine, cytidylic acid, uracil, uridylic acid, thymine) did not permit growth in EGH and added nothing to 11AA. However, combinations of purines and pyrimidines showed conclusively that the pyrimidines could take the place of the missing Factor III in the 11AA. Tables I and II show that guanine is the most active of the nucleic acid derivatives in providing Factor I activity while cytidylic acid and uracil or uridylic acid are nearly equally effective for Factor III activity.

The toxicity of adenine and hypoxanthine could be overcome by reducing the concentrations to one tenth that employed for the other purines, but they were very low in Factor I activity. Guanine nucleoside was equal to guanine. The degree of hydrolysis had an effect on the activity of cytidylic acid. Cytidine was less active than the nucleotide but more active than cytosine.

When Factor I activity was supplied by guanine (or guanosine) and Factor III activity was supplied by either uracil or cytidylic acid, the growth, while good (approximately 100,000 ciliates per ml.) was below that obtained when hydrolyzed nucleic acid was used. It was found that the best growth under these conditions could be obtained by the additions to the base media plus Factor II of both guanine and adenylic acid for Factor I activity, and both cytidylic acid and uridylic acid (or uracil) for Factor III activity. The fact that this combination never resulted in maximum growth as compared to the

TABLE II

Supplements	Base media	
	EGH + Factor II*	IIAA + Factor II*
Adenylic acid + guanine	94,000	140
Adenylic acid + guanosine	90,000	100
Adenylic acid + xanthine	20,000	70
Guanine + xanthine	100,000	110
Adenylic acid + cytosine	15,000	11,500
Adenylic acid + cytidine	19,000	10,000
Adenylic acid + cytidylic acid	17,000	15,000
Adenylic acid + uracil	16,000	17,000
Adenylic acid + uridylic acid	14,000	15,000
Adenylic acid + thymine	14,500	16,500
Guanine + cytosine	76,000	54,000
Guanine + cytidine	83,000	97,000
Guanine + cytidylic acid	78,000	110,000
Guanine + uracil	76,000	91,000
Guanine + uridylic acid	84,000	99,000
Guanine + thymine	71,000	50,000
Guanosine + cytosine	68,000	61,000
Guanosine + cytidine	79,000	93,000
Guanosine + cytidylic acid	83,000	113,000
Guanosine + uracil	72,000	98,000
Guanosine + uridylic acid	79,500	105,000
Guanosine + thymine	64,000	48,000
Adenylic acid + guanine + cytidylic acid	110,000	124,000
Adenylic acid + guanine + uridylic acid	100,000	115,000
Guanine + cytidylic acid + uridylic acid	92,000	125,000
Adenylic acid + guanine + cytidylic acid + uridylic acid	101,000	174,000

hydrolyzed nucleic acid, indicates that either the nucleic acid was contaminated with stimulating materials or that the relative concentrations of the purine and pyrimidine derivatives was more nearly optimum. The quantitative work on these substances is an important phase of the problem yet to be investigated.

### DISCUSSION

When it became clear that nucleic acid could take the place of virtually all of the Factor I and Factor III activity, it could have been predicted from previous work (1, 2, 4) that Factor I was repre-

\* The figures represent the number of ciliates per ml. in the third serial transplant after 72 hours growth.

sented by the purines and Factor III by the pyrimidines. Strong acids destroy purines and it was noted earlier (5) that hydrolysis of gelatin destroyed factors necessary for the growth of *Tetrahymena* but that the hydrolysate contained Factor III (4). The pyrimidines would not have been destroyed with this treatment.

Another line of evidence which again identifies Factor III with pyrimidines was obtained by the use of *Lactobacillus casei*. When this organism is grown in the amino acid medium of Hutchings and Peterson (11), the addition of uracil was extremely stimulatory during the first 24 hours of growth and this stimulation could be equalled by the addition of gelatin hydrolysate. It was further found that growth of *Tetrahymena* could be obtained in 11AA plus 0.05 mg. per ml. of uracil when supplemented by a water extract of timothy hay which contains subminimal amounts of Factor III (4).

While the question of the chemical nature of both Factor I and Factor III appears to be settled by these results there are a number of remarks which should be made in addition to those already given. When the original announcements of Factors I and II (1,2) appeared, folic acid was not used as a supplement. We now know that folic acid was one of the constituents of the original Factor I, for *Tetrahymena geleii* W requires exogenous folic acid for growth (the details of this work are to be published later). With the crude preparations of Factor I first used, folic acid would have been expected to be present with it. Therefore, it would probably be accurate to say that Factor I (as described earlier, 1, 2) can now be identified with certain purines forming one part, and folic acid forming the other.

The nature of Factor II is still to be elucidated. From its properties it resembles in every respect GPF-3 of Woolley and Sprince (12) and in all but one respect "streptogenin" of Woolley (13), and Woolley and Sprince (14). Thus, it is not precipitated by lead acetate and is adsorbed on activated charcoal with difficulty. Streptogenin is, according to Woolley (13), precipitated as the barium salt from certain solutions. We have repeated the techniques described for this precipitation and find that all of the Factor II activity remains in the filtrate after treatment with Ba(OH)<sub>2</sub>. It may be that Factor II can be identified with GPF-3 but it probably differs from streptogenin.\*

\* It may be that variations in the lots of starting material (Liver Fraction L) could account for this difference. In a personal communication from Dr. D. W. Woolley it was stated that the purity of the streptogenin concentrate at the time

Woolley and Sprince (12) point out that while the properties and occurrence of streptogenin and GPF-3 are identical as far as their experiments went this may be merely coincidental and need not mean chemical identity.

With the chemical nature of Factors I and III now known and the necessity for the inclusion of Factor II again proven, the doubts of the existence of "new growth factors" for the ciliate *Tetrahymena gelei* expressed by Hall (15) can be fully discounted. These doubts were admittedly on theoretical grounds and the experimental evidence here presented proves them to have been without foundation.

#### SUMMARY

Evidence is presented for the chemical nature of two essential growth factors for the ciliate *Tetrahymena gelei* W. It is concluded from the results that the original Factor I (1, 2) can now be identified with the purine derivatives of nucleic acid forming one part and folic acid forming the other. Of the purine derivatives guanine is the most important, although adenylic acid and xanthine are somewhat active singly and show a slight additive effect in the presence of guanine. Guanine nucleoside is as active, but no more so, than guanine.

Evidence is presented to show that Factor III (4) can now be identified with the pyrimidine derivatives of nucleic acid. Cytidylic acid is more active than cytidine which is in turn more active than cytosine. Uracil and uridylic acid are nearly equal in activity.

Best growth, under the conditions of these experiments, resulted when 11AA was supplemented with Factor II (from Liver Fraction L), adenylic acid, guanine, cytidylic acid and uracil (or uridylic acid). This growth was not so good, however, as when the purines and pyrimidines were replaced by hydrolyzed nucleic acid. This is taken to mean that the nucleic acid either contains unidentified stimulants or that the proportions of the derivatives are more nearly optimum.

From the chemical properties of Factor II it is concluded that it is similar to the guinea pig factor (GPF-3) of Woolley and Sprince (12) but probably not identical with streptogenin (13, 14).

of treatment with  $\text{Ba}(\text{OH})_2$  influenced to a large degree whether or not the active material would be precipitated. This question must remain open pending further investigation, particularly the use of streptogenin as a substitute for Factor II.

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# On a Synthetic Medium for the Production of Penicillin \*

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Received July 30, 1945

## INTRODUCTION

Study of the mechanism by which penicillin is formed made it advisable to develop a synthetic medium which would give maximum yields of the antibiotic.

It has been known for some time that corn steep liquor (CSL) contains a substance which enhances production of penicillin. Corn steep liquor has been fractionated in order to determine, if possible, the compound or compounds responsible for the increased yields.

The results of these studies have yielded a synthetic medium on which relatively high titers of the antibiotic have been obtained.

## EXPERIMENTAL

*Methods of Assay:* The various fractions of CSL were assayed in 16 oz. Blake bottles containing, in addition to the fraction, the mineral salt solution described by Foster *et al.* (1) modified to contain 1.5% glucose and 1.5% lactose. This medium was found to give more luxuriant growth than the use of 3% lactose as a carbon source.

An aliquot of each fraction equivalent to 8 g. of CSL was used for assay, made to 100 ml. with the basal salt and sugar solution, adjusted to pH 5.7-5.8 and sterilized at 15 lbs. pressure in an autoclave for 20 minutes. A four or five day old spore culture of the N. R. R. L. 1249 B 21 strain of *P. notatum* grown on a lactose agar slant was washed with 10 ml. of sterile distilled water, the spores removed by gently scraping the surface of the agar with a platinum loop and the resulting spore suspension transferred to a sterile DeVilbis nasal aspirator. By means of the aspirator a fine spray of spores was transferred to the surface of the medium to give a dense, smooth mat of mycelium. Samples were taken daily from the 6th day after inoculation, and assayed for penicillin by the Oxford cup method (2). Controls of the basal medium and basal plus 8 g. of CSL were run with each series of tests to determine the relative activity of each fraction.

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\* Supported in part by a grant from the Upjohn Company, Kalamazoo, Mich.

*Fractionation procedures:* Precipitation of CSL with three volumes of 95% ethyl alcohol removed approximately 50% of the dry weight without appreciable loss of the active material. Table I shows the relative activities of the alcohol filtrate and precipitate as compared with the original CSL. Since this procedure offered a rapid means of removing much of the dry weight all further fractionation was carried out on the alcohol soluble fraction unless otherwise stated.

TABLE I

*The Effect of Ethyl Alcohol Precipitation on the Stimulatory Activity of CSL*

Medium	Penicillin Titer in Oxford Units			
	5 days	6 days	7 days	8 days
Basal	25	25	20	15
Basal + 8% C.S.L.	178	205	205	240
Basal + Alcohol ppt.	77	88	88	90
Basal + Alcohol filtrate	103	205	205	210

Initial pH 5.7-5.8

The active material in the alcohol filtrate was not destroyed by 6 hour hydrolysis with 30%  $H_2SO_4$  or 30% NaOH. However, when CSL was hydrolyzed either with acid or alkali, some loss in activity occurred, indicating perhaps the formation of some toxic substance or destruction of the active material because of some substance which was removed by alcohol precipitation (*cf.* discussion).

Steam distillation and ether extraction for 48 hours from either acid or alkaline solution removed none of the activity; hence it was concluded that fatty acids, esters or alcohols were not responsible for the stimulatory action. Copper-lime treatment of the alcohol filtrate also had no appreciable effect. This result and the absence of any effect of hydrolysis seemed to remove the possibility of a carbohydrate or poly-hydroxy alcohol being responsible for the stimulation.

Addition of an excess of picric acid in alcohol to the active alcohol filtrates yielded a mass of bright yellow crystals after standing 10-12 hours in the cold. These were filtered off, washed with 95% alcohol and recrystallized from distilled water. They showed no definite melting point but charred above 200°C. The crystalline material was decomposed with  $H_2SO_4$  and extracted with ether until no picric acid remained in the aqueous phase. The ether residue was then treated with  $Ba(OH)_2$  to remove the sulfate ion and the *sulfate-free* material was assayed for

activity. Although (Table II) there is some loss of activity in the decomposed picric acid precipitate no stimulation appears in the filtrate. The decomposed precipitate when treated with ninhydrin gave a strong purple coloration. As it showed no characteristics of homo-

TABLE II  
*The Effect of Picric Acid Precipitation on the Active Material in CSL*

Medium	Penicillin Titer in Oxford Units		
	6 days	7 days	8 days
Basal	25	25	20
Basal + 8% C.S.L.	150	178	200
Basal + Picric Acid Filtrate	16	30	30
Basal + Picric Acid ppt.	27	77	105

geneity, it was thought to be a mixture of nitrogenous substances, probably amino acids. CSL was therefore extracted with butyl alcohol according to the method of Dakin (3). The results of this extraction are shown in Table III.

TABLE III  
*The Effect of 150 Hour Butyl Alcohol Extraction on the Active Material in CSL*

Medium	Penicillin Titer in Oxford Units		
	6 days	7 days	8 days
Basal	16	16	20
Basal + 8% C.S.L.	178	205	205
Basal + Butyl Alcohol Extract	30	40	40
Basal + Butyl Alcohol Residue	40	77	150

By continued extraction of a hydrolyzed zein (4) with butyl alcohol for long periods of time at a neutral pH, Dakin has shown that arginine, histidine, aspartic acid, glutamic acid and hydroxy-glutamic acid are found in the residue. As nearly all the activity remained in the residue in our experiments, we assayed four of these compounds (as the synthetic hydrochlorides) together and in various combinations before fractionating further. Lysine was also tried for activity. These data, summarized in Table IV, indicate that the activity of CSL can be replaced in large measure by a mixture of 30 mg. of histidine, 30 mg. of aspartic acid and 400 mg. of glutamic acid per 100 ml. of medium. To further test the hypothesis that amino acids are to a large extent responsible for the stimulatory effect of CSL the following experiment was performed.

TABLE IV  
*The Effect of Various Concentrations of Synthetic Amino Acids on Penicillin Production*

Basal	CSL	Histidine	Arginine	Glutamic Acid	Aspartic Acid	Lysine	CSL Extract		Penicillin Titer in Oxford Units		
							ml. HNO <sub>3</sub> treated	ml. Not treated			
ml.	g.			mg.			ml.	ml.	7 days	8 days	9 days
100	8								20	25	20
100		10							165	250	250
100		30							40	40	40
100		50							50	50	45
100		90							20	50	40
100			10						0	10	0
100			30						0	0	0
100			50						10	25	40
100			90						6	10	25
100				100					0	17	33
100				200					0	25	20
100				400					17	50	45
100				500					50	88	77
100					10				15	25	50
100					30				10	10	12
100					50				10	10	6
100					90				12	20	17
100						10			0	10	0
100						30			0	6	25
100						50			10	25	40
100						90			6	12	25
100		10	10	400					0	10	10
100		20	20	400					40	68	90
100		30	30	400					103	120	120
100		50	50	400					120	165	150
100							4		103	150	140
100								4	10	20	17
100									103	150	200

Initial pH 5.7-5.8

Two hundred g. of CSL were adjusted to pH 9 with NH<sub>4</sub>OH, precipitated with 400 ml. of 95% ethyl alcohol and filtered. The filtrate was divided into two equal parts. One-half was used as a control and the second was treated with 2.5 g. of NaNO<sub>2</sub> and HCl to a pH of 4.0. The mixture was allowed to stand at room temperature for four hours with intermittent shaking. An excess of hydroxylamine was then added to remove excess nitrite and the residual hydroxylamine removed by the addition of diacetyl

and  $\text{NiCl}_2$ . The mixture was allowed to stand in the cold for 10–12 hours and the nickel dimethylglyoximate filtered off. Residual diacetyl was then fractionally distilled from the solution and the excess  $\text{NiCl}_2$  removed by treatment with  $\text{H}_2\text{S}$ .  $\text{NaOH}$  was added to neutralize the solution and an aliquot tested for the presence of amino acids by the Van Slyke gasometric method and with ninhydrin. Both tests were negative.

Assays were performed on both the nitrous acid treated material and the untreated portion. The results (Table IV) show that the treatment had destroyed the active substances. Since nitrous acid is known to oxidize amino acids, it would appear that our hypothesis has been substantiated.

Since acetic and succinic acids were found stimulatory \* these were incorporated into the medium with resulting stimulation (Table V).

TABLE V  
*The Effect of Acetic and Succinic Acids with Synthetic Amino Acids on Penicillin Production*

Basal	CSL	Histidine	Arginine	Glutamic Acid	Acetic Acid	Succinic Acid	Penicillin Titer in Oxford Units		
							7 days	8 days	9 days
ml.	g.	mg.	mg.	mg.	u.	g.	0	0	0
100							225	250	200
100	8				.25		0	0	0
100					.5		0	0	0
100					1.0		0	0	0
100						.75	13	10	25
100						.5	20	50	50
100						1.0	60	78	80
100						1.0	0	0	0
100		30	30	400		1.0	178	205	205

Initial pH 5.7–5.8

The effect of the 1% succinic acid added is apparent although, on the other hand, acetic acid appears to have no effect under our conditions. Since the stimulatory effects reported were found with submerged growth and a different strain of mold, the discrepancy may perhaps be explained.

*Further characteristics of the active material:* The active component was found to be readily dialyzable and was completely lost from aqueous solution after a 24 hour dialysis against running water.

\* Drs. M. A. Farrell and R. W. Stone—personal communication.

Various heavy metals have also been used as precipitating agents. Mercury, as used in the Neuberg precipitation removed a large percentage of the activity. Silver and barium showed little or no effect when used on crude fractions.

### DISCUSSION

The mechanism of penicillin formation by *P. notatum* is not as yet understood. It would appear, however, that in some way this mold is perhaps able to convert one or several of the amino acids to a part of the penicillin molecule through its metabolic processes since it is known that penicillin is an organic nitrogen-containing compound. It appears that a rather delicate balance exists between the penicillin formed and the amino acid supplied. Too large a quantity causes inhibition of the production of the antibiotic. Although no explanation is apparent, it is well known that for maximum production of penicillin many environmental factors such as pH, redox potential, sugar concentration, and salt concentration must be rigidly controlled. That hydrolyzed corn steep liquor is inhibitory to penicillin production as compared to the hydrolyzed protein-free liquor, may be ascribed to the large increase in free amino acids.

The possibility of more readily extracting a chemically purer preparation of penicillin from a synthetic medium would seem to be indicated as the usual media are composed of many substances which undoubtedly interfere with its isolation.

On any medium the titer of penicillin reaches a maximum and then declines. This can best be explained by a change in the metabolism of the mold causing cessation of penicillin production rather than by assuming that the material is toxic, as up to 450 Oxford units of penicillin per ml. appear to have no effect on the growth of the mold. If such is indeed the case, there seems to be no reason why the mold cannot continue to produce the substance indefinitely.

If it is true that the production of penicillin is brought about by the influence of a substance or substances such as amino acids, then it follows that if one could maintain the metabolism of the mold at the state in which maximum yields of penicillin are produced, and continue to feed to it the substances required a constant supply of penicillin should be obtained. Such indeed seems to be the case in work now in progress in this laboratory.

### SUMMARY

1. The production of penicillin by *P. notatum* on a chemically defined medium is indicated giving 80–90% of the titer which is obtained on the usual corn steep liquor medium.
2. Corn steep liquor has been fractionated and some characteristics of the stimulatory material have been determined. It is soluble in 60% alcohol, not hydrolyzed in 30% H<sub>2</sub>SO<sub>4</sub> or 30% NaOH. It is precipitated to a large extent by picric acid and not extracted by butyl alcohol after 180 hrs.
3. The amino acids appear to be responsible, at least in part, for the stimulatory activity of corn steep liquor.
4. Three amino acids, arginine, histidine and glutamic acid in concentrations of 30, 30, and 400 mg. per 100 ml. appear to provide a large percentage of the stimulatory activity found in corn steep liquor.

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# Growth Requirements of *Penicillium Digitatum*<sup>1</sup>

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Received June 18, 1945

## INTRODUCTION

The common green mold, *Penicillium digitatum*, has been reported to cause epinasty of potato plants (1) and to stimulate the respiration and coloration of lemons (2). These effects were apparently due to ethylene emanating from the fungus, and were obtained when cultures were grown either on fruits or on potato agar. A study of the growth requirements of the organism has been undertaken preliminary to studying the formation of ethylene by *P. digitatum* grown on media of known chemical composition.<sup>2</sup>

*P. digitatum* has long been reported difficult or impossible to culture on synthetic media (3, 4), although it grows readily on a variety of media containing extracts of natural materials, including orange peel and juice (4), beerwort (5, 6), malt (7), milk (5), potato (8), and peptone (9). Since many molds and bacteria are known to require an external source of vitamins, it seemed probable that these extracts supplied growth factors not present in the synthetic media. This hypothesis might also explain reports of the stimulatory effect of other fungi upon *P. digitatum* (10, 11, 12).

Preliminary tests indicated that thiamin is essential to growth, and that biotin and, to a lesser extent, pyridoxine and calcium pantothenate definitely stimulate growth.

<sup>1</sup> Published with the approval of the Monographs Publication Committee, Oregon State College. Research paper No. 95, School of Science, Department of Chemistry.

This work was supported by Research Corporation, Inc.

<sup>2</sup> The problem was undertaken at the suggestion of Elmer Hansen, Oregon Experiment Station.

### METHODS AND MATERIALS

*Organism.* The organism used was *Penicillium digitatum* Sacc., isolated from a lemon fruit. Stock cultures of the mold were grown on potato-glucose agar slants at room temperature for about a week, and then stored in the refrigerator.

*Inoculum.* Inoculum was prepared by pouring several portions of sterile distilled water over a stock slant and then through a thin layer of sterile glass wool to remove large particles of mold or agar. Half ml. portions of this spore suspension were then transferred by means of a sterile pipette to the medium in the culture flasks described below.

*Basal Medium.* The medium employed had the following composition:  $\text{KH}_2\text{PO}_4$ , 1.0 g.,  $\text{NaNO}_3$ , 1.0 g.,  $\text{MgSO}_4$ , 0.25 g.,  $\text{KCl}$  0.10 g.,  $\text{CaCl}_2$ , 0.10 g.,  $\text{FeCl}_3$ , 5.0 mg.,  $\text{MnCl}_2$ , 0.10 mg.,  $\text{ZnCl}_2$ , 0.05 mg.,  $\text{H}_3\text{BO}_3$ , 0.05 mg.,  $\text{CuCl}_2$ , 0.01 mg.,  $\text{KI}$  0.01 mg., sucrose 25 g., asparagin 3 g., ammonium tartrate 5 g., casein hydrolysate 40 ml., water and supplements to make 1 liter. The pH was adjusted to 3.0.

For the preparation of casein hydrolysate, 50 g. of vitamin-free (Labco) casein were mixed with 250 ml. of 25% sulfuric acid, and autoclaved for 10 hours at 15 pounds pressure. The sulfuric acid was removed with barium hydroxide, any excess barium ion being carefully removed with sulfuric acid. The solution was autoclaved 10 minutes, filtered, and adjusted to contain 100 mg. of dry matter per ml. It was preserved under toluene (13).

*Orange Rind Extract.* Five g. of thinly pared yellow rind were added to 50 g. of water, and steamed for 15 minutes. The extract was cooled and filtered.

*Growth of Cultures.* The mold was grown in 25 ml. portions of basal medium in cotton-plugged 125 ml. Erlenmeyer flasks, sterilized by autoclaving for 15 minutes at 15 pounds pressure. The cultures were incubated at 25°C., the approximate optimum for this mold (12, 14, 15, 16). After 70–72 hours' growth, the mold was killed by steaming for five minutes, cooled, separated from the medium by filtering through weighed Gooch crucibles containing a disc of coarse filter paper, thoroughly washed, dried 5–12 hours at 70°C., and weighed. The 72-hour growth period was selected because it permitted maximum growth before the onset of excessive autolysis in the most rapidly growing cultures.

Determination of growth by measurement of the diameter of colonies on agar plates proved unsatisfactory, as growth was often manifested by thickening of the colony rather than by increase in area.

### EXPERIMENTAL RESULTS

*Reliability of Results.* In general each experimental value listed in the tables is an average of two or more determinations. In deficient media, 95% of replicate determinations agreed within  $\pm 10\%$ . Agreement was much poorer, however, in fairly adequate media, where the balance among environmental factors appeared to be extremely delicate. This limited the use of the organism for assay purposes.

*Carbon Sources.* The basal medium was enriched by the addition of

10  $\gamma$  thiamin hydrochloride, 20  $\gamma$  pyridoxine hydrochloride, 15  $\gamma$  calcium pantothenate, and 0.01  $\gamma$  biotin per flask. Sucrose and ammonium tartrate were omitted. A small amount of carbon was available in the medium due to the presence of casein and asparagin.

Carbon sources were added in a concentration of 375 mg. per flask (15 g. per liter). This amount does not permit optimum growth, but was used in order to avoid the addition of large quantities of sodium hydroxide in neutralizing the media containing organic acids, since sodium ion has shown some indication of toxicity for this organism. Results are given in Table I.

TABLE I  
*Effect of Carbon Source on Growth of *P. digitatum* at pH 3.0*

Carbon source (375 mg./25 ml.)	Av. dry wt. of mat (mg.)
Blanks	7
Sucrose	117
Glucose	130
Fructose	151
Galactose	96
Acetic acid	0
Citric acid	40
Malic acid	17
Oxalic acid	3
Tartaric acid	3
Ammonium tartrate	1

Of the acids tried, only citric and malic supported appreciable growth, in neither case comparable with that obtained with sugars. These results are in general agreement with those of Kursanov and Alekseeva (17). Camp (9) and Marloth (4) obtained no growth with citric acid alone, but found that growth was increased by its addition to media containing sugar. Marloth suggested that the increased growth was most probably due to better pH control.

*Nitrogen Sources.* The basal medium was modified by replacing sodium nitrate with sodium chloride, omitting ammonium tartrate, casein and asparagin, and using 15 g. of sucrose as a carbon source. The medium was enriched with 10  $\gamma$  thiamin, 20  $\gamma$  pyridoxine, 15  $\gamma$  pantothenate, and 0.01  $\gamma$  biotin per flask. A total of 200 mg. per flask (8 g. per liter) of the nitrogen-containing supplements was used. Results are given in Table II.

Moderate growth was supported by ammonium salts, aspartic acid or peptone; casein or asparagin permitted better growth, but a mixture of casein and asparagin was better than either substance alone. Peptone added to the medium at the expense of half the asparagin was unable to substitute for the latter.

TABLE II  
*Effect of Nitrogen Source on Growth of *P. digitatum* at pH 3.0*

Nitrogen source (200 mg./25 ml.)	Av. dry wt. of mat (mg.)	Appearance
None	0	
KNO <sub>3</sub>	0	(White or pale green)
NH <sub>4</sub> NO <sub>3</sub>	12	except where otherwise noted)
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	50	
Ammonium tartrate	51	
Urea	5	Slimy, submerged
Glycine	13	
Aspartic acid	36	Very dark green
Aspartic acid, (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	46	
Peptone	32	
Casein	67	Submerged
Asparagin	102	Medium bright yellow
Asparagin, casein (100 mg. each)	142	Medium bright yellow
Asparagin, peptone (100 mg. each)	72	Medium bright yellow

At pH 3, the utilization of ammonium ion, even from ammonium tartrate, resulted in a drop in pH to a level unfavorable for growth (2.2-2.4). However, the relative growth on ammonium salts was no better in media of initial pH 4.5 or 6.5, which remained within a favorable pH range throughout the experiment.

The nitrate ion exhibited an inhibitory effect. Ammonium nitrate produced less growth than the other ammonium salts tried, and in other experiments its addition to casein-asparagin media resulted in a reduction of growth.

These results are in general agreement with those obtained by Marloth (4). Kursanov and Alekseeva (17), however, obtained no growth on ammonium sulfate or nitrate, fair growth on glycine, asparagin or potassium nitrate, and excellent growth on peptone. Wöltje (6) also obtained fair growth on potassium nitrate. The reasons for these discrepancies are unknown, but they may be due to strain differences.

**Vitamins.** Thiamin was found to be essential for the growth of *P. digitatum*. Good quantitative curves were obtained for the relationship of growth to the amount of thiamin present, as shown in Fig. 1. With concentrations of thiamin above 1 γ per 25 ml., sporulation seemed to be retarded in favor of very heavy mycelial growth.

Thiazole was utilized as well as thiamin, on an equimolecular basis, but the pyrimidine component appeared to be synthesized by the organism, since its addition to media containing thiazole did not improve growth (Fig. 1).

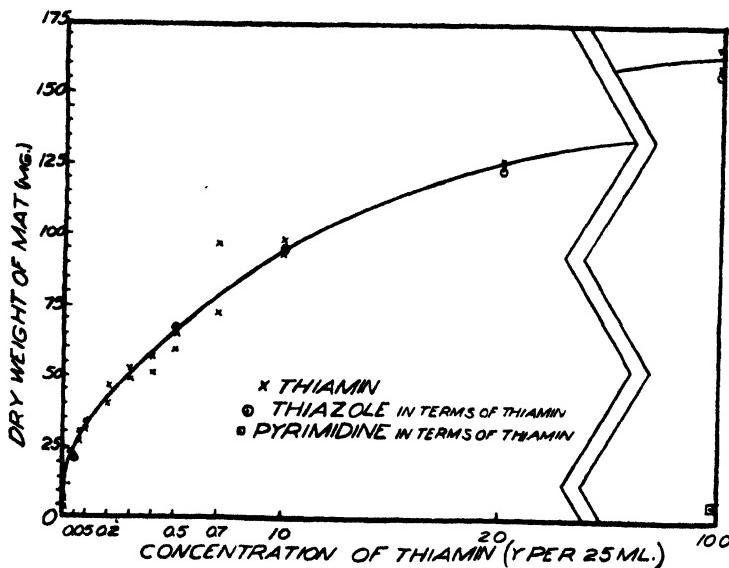


FIG. 1  
Effect of Thiamin and Thiazole on Growth of *P. digitatum*

Pyritthiamin inhibited the utilization of thiamin and cocarboxylase by the organism in the same manner as was shown for *Lactobacillus fermenti* (18).

When adequate thiamin was present, pyridoxine, pantothenate and biotin produced some stimulation of growth. Cultures containing added biotin sporulated earlier and more abundantly than those lacking this factor. In the presence of adequate amounts of these vitamins, growth on the basal medium was found to be very nearly as great as on media containing added orange rind extract. The

stimulation obtained by the addition of orange rind extract may be largely due, in fact, to the thiamin present. Orange rind was found to have a thiamin content of  $6 \gamma$  per gram, while orange juice has been reported to contain  $1 \gamma$  of thiamin and  $0.02 \gamma$  of biotin per ml. (19). The high thiamin content of the epi- and mesocarps of citrus fruits together with the high thiamin requirement and biotin tolerance of the mold may account for its preferential growth on these fruits.

TABLE III  
*Effect of Carbon Source on Growth of *P. digitatum* at pH 6.5*

Carbon source (375 mg./25 ml.)	Av. dry wt. of mat (mg.)
Sucrose	65
Glucose	55
Fructose	75
Galactose	61
Maltose	9
Acetic acid	3
Citric acid	2
Malic acid	2
Oxalic acid	3
Tartaric acid	2
Ammonium tartrate	3

*pH of Medium.* The growth curves of the organism at different initial pH values showed considerable variation in limits and in location of optima. However, little or no growth was observed below pH 2.0, or above 7.6. The curves were consistently bimodal, with a peak near 3.0–3.5, and another lesser peak at or slightly above pH 6.0.

In all cultures, growth was accompanied by slightly increased acidity. From an initial value of 6.0, the pH was lowered approximately two units. The change was relatively small in the more acid range (0.3–0.5 units).

Using ammonium sulfate as a nitrogen source, Marloth (4) found an optimum pH range of 3–6, with a probable peak near 4.5. However, the utilization of ammonium ions produces a considerably greater drop in pH during growth (from 4.4 to 2.7; 6.4 to 3.3) than is produced with asparagin and casein (4.4 to 3.8; 6.4 to 4.4). The initial pH required to produce optimum growth would thus be higher in an ammonium sulfate medium.

*Growth at pH 6.5.* In contrast to the results obtained at pH 3.0, growth at the less favorable pH of 6.5 or higher was found to be

markedly stimulated by orange rind extract. The nutritional requirements of the organism were therefore investigated at the higher pH value.

Tables III and IV summarize the results of experiments on carbon and nitrogen sources, using the same media as described previously for pH 3.0.

TABLE IV  
*Effect of Nitrogen Source on Growth of P. digitatum at pH 6.5*

Nitrogen source (200 mg./25 ml.)	Av. dry wt. of mat (mg.)
None	2
KNO <sub>3</sub>	1
NH <sub>4</sub> NO <sub>3</sub>	18
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	26
Ammonium tartrate	20
Urea	5
Glycine	2
Aspartic acid	9
Peptone	33
Casein	33
Asparagin	33
Asparagin, casein (100 mg. each)	65

The growth response curve for thiamin at pH 6.5 shows the same form and maximum as at pH 3.0, although the total growth is much lower. Thiazole can replace thiamin at this pH as well. The general shape of the thiamin curve is unaffected by varying amounts of pyridoxine, pantothenate and biotin.

At and above pH 6.5 pyridoxine, pantothenate and biotin are much more stimulatory than in the lower pH range. Biotin actually extends the upper limit of growth, as may be seen from Table V. Some of the

TABLE V  
*Effect of Biotin on Growth at Different pH Values*

Biotin per 25 ml.	Init. pH 3.0	6.0	6.5	7.0	7.4	7.6
None	142	71	13	6	0	0
0.01γ	120	118	92	57	28	2

results obtained at this pH indicated a possible biotin-sparing action of pyridoxine and pantothenate, but this could not be confirmed.

The changing requirements for growth of this mold at different pH values are of interest. A correlation of these requirements with possible

variations in the end-products, both in this and other organisms, might provide a means of studying the role of various nutrients in metabolism. In this connection the work of Stokes, Foster, and Woodward (20), who concluded that a "pyridoxinless" X-ray mutant of *Neurospora sitophila* could synthesize pyridoxin only within a limited pH range and in the presence of ammonium ion, may be mentioned.

*Evidence for an Unknown Factor in Orange Rind.* In the presence of optimal amounts of the above four vitamins, at an initial pH of 6.8, the addition of orange rind extract, also at pH 6.8, equivalent to 200 mg. of fresh rind per flask, increased growth approximately 250%. This stimulation appeared to be due to an unidentified factor or factors. No effect was produced by the following supplements: acetic, citric, fumaric, lactic, maleic, pyruvic or succinic acids, tryptophane, arginine, cystine, methionine, nucleic acid, ammonia-hydrolyzed nucleic acid, adenine, guanine, xanthine, hypoxanthine, uracil, ascorbic acid, hesperidin, hesperidin chalcone, hesperidin methyl chalcone, quercitrin, riboflavin, nicotinic acid, inositol, p-aminobenzoic acid, choline, folic acid, xanthopterin or the *L. casei* factor of Stokstad.

The active principle in the orange rind was soluble in water, methanol, 50% ethanol, and 50% acetone. It was insoluble in 95% ethanol, acetone, dioxane, ether, and pyridine. It was stable to drying at 105°C. for eleven hours, to autoclaving for three hours at pH 1 and pH 5, and to bromination, but was destroyed by ashing, by autoclaving at pH 11, by hydrogen peroxide in acid or alkali, and by nitrous acid. It was removed by dialysis.

Concentration attempts were generally unsuccessful. The factor was not readily adsorbed by Darco G-60, Norite or Lloyd's reagent. It was partially precipitated by lead acetate or barium chloride, but could not be recovered from the salts by treatment with hydrogen sulfide or sulfuric acid. Also, separations were difficult to measure quantitatively because of the previously mentioned erratic behavior of the organism in favorable growth media.

#### ACKNOWLEDGMENT

The authors are indebted to California Fruit Growers' Exchange, Ontario, California, and to Dr. C. G. Wawra, University of Southern California, for samples of hesperidin, hesperidin chalcone, and hesperidin methyl chalcone; to Dr. E. L. R. Stokstad, Lederle Laboratories, for the *L. casei* factor; to Dr. J. J. Pfiffner, Parke-

Davis Laboratories, for xanthopterin; to Dr. R. J. Williams, University of Texas, for folic acid; and to Dr. W. J. Robbins, New York Botanical Gardens, for the hypoxanthine used. The authors also wish to thank Dr. Kenneth B. Raper, Northern Regional Research Laboratory, Peoria, Illinois, for confirming the identity of the organism, and Miss Margaret J. Bennett for the thiamin assays using *L. fermenti*.

### SUMMARY

At pH 3 (the approximate optimum for *P. digitatum*), maximum growth is obtained on a medium whose only ingredient of unknown composition is vitamin-free hydrolyzed casein. The carbohydrate requirements are well satisfied by sucrose, glucose, fructose or galactose. Poor to vanishing growth is obtained with various organic acids. The best nitrogen sources observed are asparagin and hydrolyzed cascina, especially in combination. Peptone, aspartic acid or ammonium salts support moderate growth.

The thiazole moiety of thiamin is required by the organism. Quantitative growth curves for thiamin may be obtained over the range 0.01–3 γ per 25 ml. Pyridoxine, pantothenate and biotin are stimulatory.

At and above pH 6.5 (above the second optimum for the organism), the effect of biotin is much more pronounced than at pH 3.0. In the presence of 0.01 γ per 25 ml., the upper limit of growth is extended approximately one pH unit. Growth in this pH range is also markedly stimulated by an unidentified factor present in orange rind. Various properties of this factor have been described, but its concentration has not been effected, since at this pH the organism is unsatisfactory for quantitative assay purposes.

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# The Chemical Determination of Tocopherols in Animal Fats: the Stability of Hog Fats in Relation to Fatty Acid Composition and Tocopherol Contents † \*\*

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Received July 30, 1945

## INTRODUCTION

It has been recognized by many investigators that natural antioxidants are present in the fats of hogs but the identities and quantities of such antioxidants have not been clearly determined. Ever since the early studies on vitamin E, in which vitamin E deficiency in rats was regularly produced for experimental purposes by using ordinary commercial lards in the diets (1), it has been quite generally but mistakenly believed that tocopherols are absent from hog fats, or are present in amounts too insignificant to have any importance either biologically or in relation to the keeping qualities of lards. Indeed, the absence of tocopherols has to some extent come to be regarded as a distinguishing feature between animal and vegetable fats. In recent years this belief has been enhanced by the failure of the newer analytical methods to reveal any tocopherols in most commercially prepared lards.

The first reported effort to determine chemically the presence and amount of tocopherols in hog fats seems to have been made by Karrer and Keller (2). Using a gold chloride potentiometric titration method (3) and the Emmerie and Engel method, (4), they measured reducing substances in swine fat which they reported as tocopherols, present in the amount of about 2 γ/g. of fat.

In early experiments in this laboratory, the observation that hog fats normally contain tocopherols was confirmed chemically and spectrophotometrically (unpublished data), and evidence was also obtained that the amounts are sufficient to have an important bearing on the keeping qualities. Subsequently it was shown by other methods that the stability of carefully rendered fats from rats is primarily related to their tocopherol content (5).

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† Aided by grants from the Hormel Research Foundation and the Hormel Institute.

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\*\* Presented at the 18th Fall Meeting of the American Oil Chemist's Society, Chicago, October, 1944.

As has been suggested by Riemenschneider *et al.* (6), the absence of tocopherols from most commercial lards is a result of the rendering and refining processes. The generally poor keeping qualities of steam rendered lards to which no antioxidants have been added is due in part to a destruction of tocopherols and in part to the addition of pro-oxidants such as iron and decomposition products of peroxides. Both commonly occur during commercial steam rendering.

To study the relationship between the inherent stabilities of carefully rendered hog fats and their tocopherol contents, and to conduct projected experiments with tocopherols in other animals, it became highly desirable to have a sensitive and accurate method for the quantitative estimation of the relatively small amounts of tocopherols present in animal fats. For this particular purpose none of the many biological, physical and chemical methods now available (3, 4, 7-21) appeared suitable in unmodified form. Bioassays could not be used due to the difficulties involved in concentrating without loss the required amounts of tocopherol. The available physical methods are designed primarily for the determination of larger amounts of tocopherol in purer form. Most chemical methods suffer from a lack of specificity because, in general, the reactions and color changes upon which they depend are subject to interference by other substances present in fats, some of them unidentified.

Attempts were made to adapt several of the chemical methods to the desired use. The colorimetric Emmerie and Engel method (4) is capable of measuring small quantities of tocopherols but is unreliable because an almost unlimited number of biological substances are capable of producing the red color upon which the determination depends. Devlin and Mattill (11) and Hines and Mattill (12) have introduced modifications incorporating the Parker-McFarlane treatment (22), designed to eliminate the interfering substances present in muscle tissues and other organs. Attempts to utilize these modifications in the present study were abandoned in the face of errors caused by sterols and other substances in fats. A sensitive method introduced by Seudi and Buhs (13) was likewise abandoned when interfering substances contributed to the reduction of the 2,6-dichlorophenol-indophenol solution.

The method that was finally developed and used is based on the Furter and Meyer procedure (14). In its original form, the Furter and Meyer method is neither very sensitive nor specific. The red orthoquinones produced by the nitric acid oxidation of tocopherols are only moderately chromatic. In addition, although the red color produced by oxidation with nitric acid has been said to be quite specific for 6-hydroxychromans (14, 23) a number of other biological substances frequently present in fats and other natural materials yield oxidation products with absorption spectra of such character that they interfere in a simple colorimetric determination (15, 24).

Andrews and Binnington have reported a modification of the Furter and Meyer method, developed for the estimation of tocopherols in cereals and milling products (16) and have published some of their measurements (25). The fat is extracted from the product and saponified. The unsaponifiable matter is oxidized with nitric acid, the oxidation products are chromatographed on alumina, and the red orthoquinones are separated and measured.\* Their procedure offers one distinct improvement over

\* We gratefully acknowledge the cooperation of Mr. D. S. Binnington and Dr. J. S. Andrews of General Mills, Inc., in giving us a full description of the procedure. The untimely death of Mr. Binnington prevented publication of the method, but Dr. Andrews has courteously given us permission to describe the procedure in detail.

other chemical methods in that a high degree of specificity is obtained by the introduction of the chromatographic separation of the orthoquinones. They have pointed out that although tocoquinone also yields red orthoquinones, if unsaturated glycerides are present the tocoquinone remains with the saponified matter during the extraction of the tocopherols and other unsaponifiable matter (16).

In the present study a step was added which greatly improved the sensitivity of the method and made it applicable to the determination of tocopherols in animal fats. Briefly, the added step consists in reacting the purified orthoquinones with a solution of leuco methylene blue and measuring the development of the blue color in a spectrophotometer (or photoelectric colorimeter). With this technique it is possible to measure 20 γ of tocopherol with an estimated average deviation from the mean of less than 10%. The method does not distinguish α-, β-, and γ-tocopherols.

## EXPERIMENTAL

1. *Preparation of the Fats:* No attempt was made to determine the tocopherol content of the whole adipose tissues. Rather, the fats were carefully rendered by a method described elsewhere (5), designed to cause a minimum loss and destruction of tocopherols.

2. *Procedure for Determination of Tocopherols in Fats:* The analytical procedure for the determination of tocopherols may be conveniently divided into three steps: (a) saponification of the fat and preparation of the unsaponifiable fraction, (b) conversion of the tocopherols in the unsaponifiable fraction to purified orthoquinones, and (c) estimation of the tocopherols by reaction of the orthoquinones with leuco methylene blue. In the ensuing outline the description of steps (a) and (b) is taken largely verbatim from the directions given by Andrews and Binnington, containing only such additions and modifications as are necessary to adapt the method to the estimation of tocopherols in animal fats and to accommodate step (c). The apparatus in Fig. 1 was designed by Binnington.

### (a) *Saponification and Preparation of Unsaponifiable Fraction*

Since the tocopherols are sensitive to oxidation in the presence of alkalies, saponification must be conducted with air-free alkali in an inert atmosphere that must be maintained throughout the subsequent extraction of the unsaponifiable matter.

Air-free alkali is prepared by dissolving 400 g. of KOH pellets in two l. of absolute methanol in an Erlenmeyer flask, using a stream of N<sub>2</sub> to obtain the necessary agitation. The solution is filtered under suction through a sintered glass funnel to remove insoluble carbonates and then transferred under N<sub>2</sub> into the storage bottle illustrated in Fig. 1, c. A gentle stream of N<sub>2</sub> is passed through the delivery tube in a reverse direction for one hour to insure complete removal of oxygen. N<sub>2</sub> pressure for delivery of the reagent is applied by means of a rubber balloon, previously filled with N<sub>2</sub>. This balloon may be refilled when necessary through the side stopcock.

The safest procedure is to purify the N, used in this and all subsequent operations by passage through Fieser's reagent (26) or over a heated copper gauze. It is highly important that all possible precautions be taken to completely prohibit contact of the tocopherols with oxygen, particularly when alkali is present.

Saponification and extraction of the unsaponifiable matter is carried out in the apparatus shown in Fig. 1, a. The condenser is removed and between 8 and 9 g. of melted fat are transferred directly into the apparatus through the top. With the condenser back in place, the system is evacuated with a good water pump. N<sub>2</sub> is

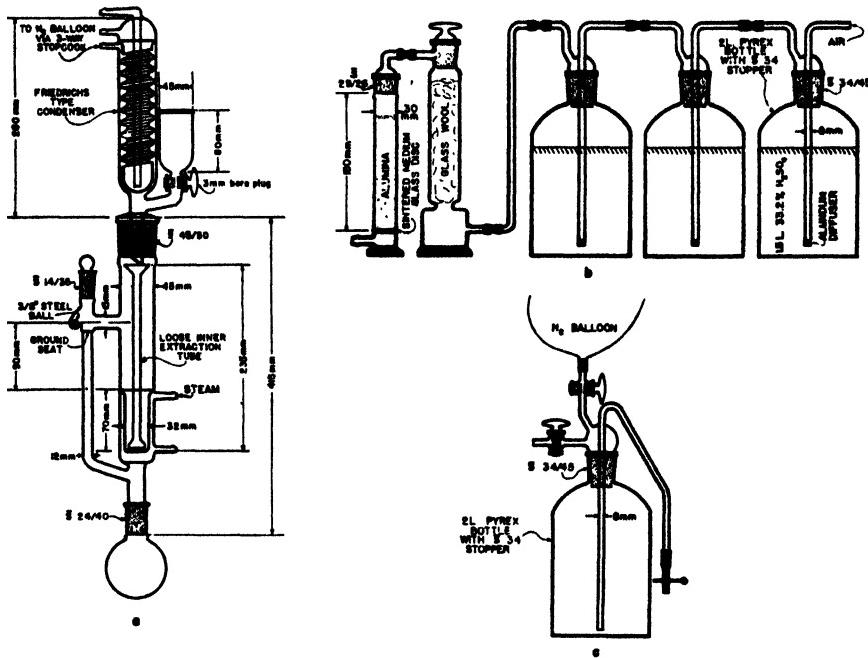


FIG. 1

Apparatus Designed by D. S. Binnington for Use in Determination of Tocopherols

admitted from a storage balloon and the evacuation is repeated. Seventeen ml. of the air-free KOH solution and 13 ml. of air-free CH<sub>3</sub>OH are added through the tap funnel with the aid of suction and the process of alternate evacuation and flushing with N<sub>2</sub> is repeated five times. (With liquid fats, as much as 10.5 g. of the fat and a corresponding increase in the amount of alkali may be used, and the addition of the excess pure methyl alcohol is unnecessary.)

With the apparatus under a slight positive N<sub>2</sub> pressure, maintained by the balloon, the steel ball valve is transferred from the side pocket to its seat with a powerful hand magnet or an electromagnet. Steam is then passed through the jacket. After 45 min-

utes, and with the steam still circulated through the jacket, 50 ml. of air-free water (stored under N<sub>2</sub>) are added slowly through the tap funnel, the apparatus being carefully vented to the air via the three-way stopcock. The steam is turned off and an additional 20 ml. of air-free water are rapidly added. The apparatus is returned to the nitrogen pressure of the balloon and the steel ball valve returned to the side pocket.

After the apparatus and contents have cooled to room temperature, and not before, peroxide-free USP ethyl ether is added until the lower flask is one-third filled. The

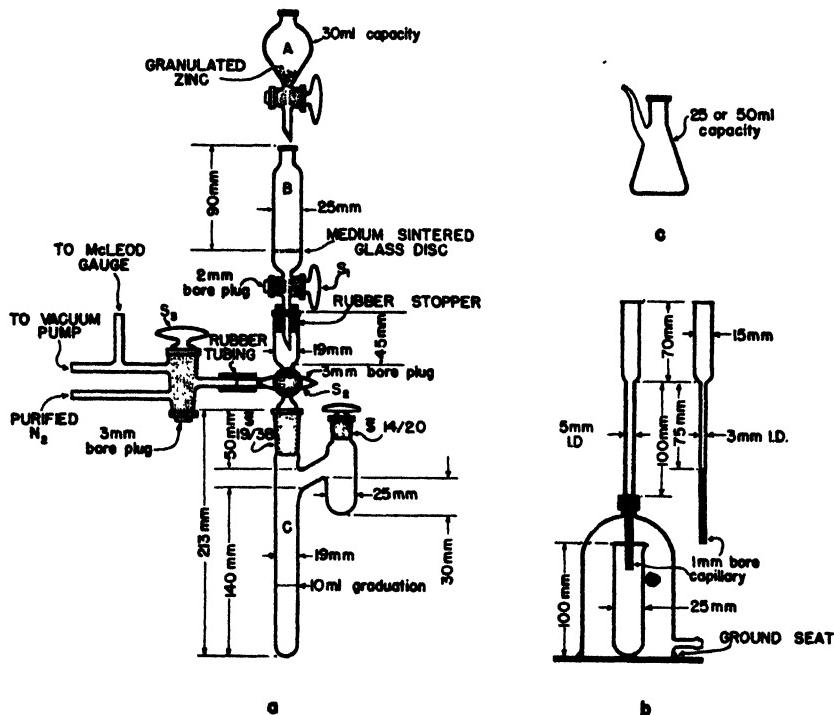


FIG. 2

## Accessory Apparatus Used in Methylene Blue Method

ether is permitted to boil on a steam bath for 3½ hours. This length of time is ample for a complete extraction if the temperature of the condenser water does not exceed 15°C. Any tendency toward bumping caused by the formation of a two phase system in the lower flask is best minimized by introducing a few ml. of absolute alcohol into the flask before the saponification is begun. A pinch of washed sand introduced at the same time promotes a smoother boiling of the ether.

The extract is transferred to a 250 ml. Squibb separatory funnel with a minimum of exposure to the air. It is immediately washed, gently at first, with air-free water until free from alkali as shown by testing the wash water with phenolphthalein. Finally, the

extract is dried over anhydrous  $\text{Na}_2\text{SO}_4$  and filtered. The  $\text{Na}_2\text{SO}_4$  is washed several times with small portions of ether and the washings are added to the filtrate.

(b) *Conversion of the Tocopherols to Orthoquinones*

The extract is evaporated to dryness, the residue being finally contained in a small transfer flask, Fig. 2, c. Two ml. of benzene are added and the sterols and carotenoids are largely removed by adsorption on purified florisil,\* using the apparatus in Fig. 2, b. The florisil is purified and used essentially according to the procedure described by Devlin and Mattill (11) except that following purification it is dried in an oven at 120°C. for 3 hours. The dimensions of the column used in this step are 5 × 100 mm. Following passage of the solution, the transfer flask and column are washed with three 2 ml. portions of benzene. The percolate is run directly into a 1 × 4 inch test tube provided with a lip for pouring. The benzene is removed on a water bath under a stream of  $\text{N}_2$ . Ten ml. of absolute n-butyl alcohol are added to dissolve the residue, warming, if necessary, to insure complete solution. When cool, 2 ml. of conc.  $\text{HNO}_3$  are added from a pipette into the center of the solution to prevent local concentration and are thoroughly mixed. The tube is immediately placed in a vigorously boiling water bath for 2 minutes ( $\pm 5$  seconds) and then quickly placed in a bath of ice water and cooled to room temperature as rapidly as possible. The time of heating is critical and the 2 minute period specified is optimum for the apparatus and conditions described.

Without delay, the reaction products are transferred with the aid of three 5 ml. portions of absolute ethyl alcohol to a 250 ml. separatory funnel containing 150 ml. of  $\text{H}_2\text{O}$ . Twenty-five ml. of low boiling petroleum ether are added, and the contents of the funnel are shaken well and permitted to stand for one hour. The lower layer is drawn off and discarded. The petroleum ether layer is washed twice with 100 ml. portions of  $\text{H}_2\text{O}$ , using gentle agitation, and then dried by filtration through a small amount of anhydrous  $\text{Na}_2\text{SO}_4$ , into a small transfer flask. The funnel and  $\text{Na}_2\text{SO}_4$  are rinsed with an additional small quantity of petroleum ether. The filtrate is evaporated to dryness on a tepid water bath (35°C.) in a stream of  $\text{N}_2$ . When the petroleum ether has disappeared, the stream of  $\text{N}_2$  is continued for 3 to 5 minutes but no longer than is necessary to remove the odor of the last traces of butyl nitrite.

The residue is taken up in 2 ml. of low boiling petroleum ether and drawn through a column of Merck's Brockman alumina (American variety) containing 12% moisture. The apparatus shown in Fig. 2, b is again used, this time with a column whose dimensions are 3 × 75 mm. The transfer flask and column are rinsed with another 2 ml. of petroleum ether. The red orthoquinone is strongly adsorbed near the top of the column immediately below a layer of impurities. The column is then carefully washed with small amounts of benzene until the red zone has almost reached the bottom of the column. When the original sample contains as little as 20 γ of tocopherol, the red zone is barely visible and care must be taken not to overlook it. The last of the benzene is drawn out and the column is washed once with petroleum ether and dried by suction to such an extent that the top of the column containing the strongly adsorbed impurities may be poured off. The remainder of the column containing the red zone is eluted with a small amount of peroxide-free ethyl ether directly into the side pocket of the

\* Minus 200 mesh, obtained from the Floridin Co., Warren, Pa.

spectrophotometer tube shown in Fig. 2, a. If it is judged that too much quinone is present for the methylene blue determination, the eluant is received in a 10 ml. volumetric flask instead and an aliquot taken for the final step.

The moisture content of the alumina is important and various types of alumina have widely different optimum moisture levels. With the American made Brockman alumina, a moisture content appreciably less than 12% results in a too strong adsorption of the quinones and more than 12% prevents adequate separation of impurities. The proper moisture content may be attained by exposing the dried alumina to the atmosphere above 33.2% sulfuric acid (sp. gr. 1.249 at 15.5°C.), either in thin layers in a desiccator or in a tower in the train shown in Fig. 1, b. In either case the moisture content of the alumina should be determined by measuring the loss in weight on ignition in a muffle for one hour at 450 to 550°C.

When relatively large amounts of tocopherol are present, as in some vegetable oils, it is possible to conclude the analysis with a colorimetric measurement of an alcohol solution of the orthoquinones, using an Evelyn colorimeter equipped with a 490 m $\mu$  filter. In such cases, larger adsorption columns and a two stage adsorption may be desirable since vegetable oils usually give rise to more pigments and other impurities than animal fats.

### (c) Reaction of Quinones with Leuco Methylene Blue

Methylene blue is dissolved in 95% alcohol to produce a few ml. of concentrated solution. After filtering, the solution is diluted with a mixture of 80 parts by volume ethyl alcohol (95%), 15 parts water, and 5 parts glacial acetic acid, to produce a stock solution of methylene blue having an optical density of approximately 2.0 to 2.5 per cm. of cell thickness at 6500 Å. Attainment of the proper concentration is achieved with the aid of spectrophotometric measurements on aliquots at still greater dilution against a blank of solvent mixture.

Fig. 2, a depicts the apparatus used in the next phase of the analysis. Fifteen ml. of the stock methylene blue solution are introduced into the separatory funnel A containing a few grams of granulated zinc. The reaction mixture is protected from oxygen by passing a gentle stream of N<sub>2</sub> up through the stem of the funnel.

While the preliminary reduction of the methylene blue is thus going on, the ether eluant containing the orthoquinones is introduced into the side pocket of the spectrophotometer tube C. The solvent is evaporated at room temperature by means of a gentle stream of N<sub>2</sub>. The application of a stream of N<sub>2</sub> and the subsequent application of vacuum to the quinones should be continued only as long as is necessary; prolonged vaporizing conditions cause low results.

In chamber B, containing a sealed in, sintered glass plate, is placed a pinch of zinc dust, and the chamber is closed by means of a rubber stopper. Stopcock S<sub>1</sub>, the three-way T-bore stopcock S<sub>2</sub>, and the three-way two-angle-bore stopcock S<sub>3</sub> are opened so that the entire apparatus is evacuated by a mechanical oil pump. When the McLeod gauge in the vacuum line shows that the pressure has dropped to 10 or 20  $\mu$ , chamber C is closed off and N<sub>2</sub> is admitted to chamber B. The stopper is removed from B, and a flow of N<sub>2</sub> into the chamber through the system of stopcocks is continued while the almost reduced methylene blue is admitted through the top from A. After thoroughly mixing the contents of B, the stopper is replaced, S<sub>1</sub> is closed, and the small chamber

above S<sub>2</sub> is evacuated. When the pressure has fallen to 20  $\mu$  or less, S<sub>2</sub> is turned to close off the line to S<sub>1</sub>, and the rubber connection between S<sub>2</sub> and S<sub>1</sub> is disconnected. S<sub>1</sub> is opened and 10 ml. of the reduced methylene blue are permitted to flow into C. S<sub>2</sub> is turned to close off C, the apparatus is removed from its support, and B is disconnected. With the side pocket in the uppermost position to avoid contact of the methylene blue with the quinones, C is tipped back and forth gently 30 or 40 times or until a constant reading is obtained in the spectrophotometer. Usually the leuco methylene blue has a transmission of more than 90% at 6500 Å when compared with 95% alcohol in square cuvettes of 1.25 cm. thickness.

The leuco methylene blue is then allowed to mix with the quinones, shaken vigorously, and a spectrophotometer reading made. Alternate shakings and readings are continued until a minimum transmission at 6500 Å is obtained. Thereafter, a fading of the blue color begins, but is so slow that no correction is required. The optical density corresponding to the minimum transmission is referred to an empirical reference curve to obtain the tocopherol content of the sample.

In spite of the fact that the procedure in this method of analysis for tocopherols has some critical points requiring considerable caution, and appears rather lengthy, one individual may conveniently complete analyses at the rate of two or three per average working day if the work is properly organized. It is believed that with slight modifications the method may be utilized in measuring tocopherols in other animal tissues.

3. *Preparation of Reference Curve:* Since there are small losses of one kind or another during the analytical process, it is necessary to prepare an empirical reference curve relating the measured optical densities to the original tocopherol contents. The data for this curve must be obtained by performing the complete analysis on samples of fat containing known amounts of tocopherol.

On the basis of equivalent concentrations,  $\alpha$ - and  $\gamma$ -tocopherols were reported to give the same orthoquinone reference curves within the limits of experimental error.\* A comparison of measurements on several relatively pure samples of  $\alpha$ -tocopherol and a mixture of 50%  $\alpha$ - and 50%  $\gamma$ -tocopherol indicates that this observation also holds true for the methylene blue reference curves, as might be reasonably expected. The much less abundantly occurring  $\beta$ -tocopherol was thought to give orthoquinone solutions having somewhat lower optical densities,\* but possibly this is attributable to the difference in the absorption maximum of the orthoquinone obtained from  $\beta$ -tocopherol (27).

Several samples of  $\alpha$ -,  $\gamma$ -, and mixed  $\alpha$ - and  $\gamma$ -tocopherols were assayed directly (without saponification) in order to select a standard of the highest possible purity. A special highly purified mixture of approximately 50%  $\alpha$ - and 50%  $\gamma$ -natural tocopherols † was found to assay 11.2% higher than the best available commercial preparation of synthetic  $\alpha$ -tocopherol. A solution of a weighed portion of this sample in petroleum ether was prepared. Aliquots of the solution were evaporated to dryness in the absence of oxygen and the residues taken up in samples of fat that were essentially free of tocopherols.

Three samples of fat were used as substrates for the tocopherol standard. Two

\* D. S. Binnington, private communication.

† Kindly supplied by the Chemical Research Division of General Mills, Inc.

samples were prepared by rendering the abdominal fats from vitamin E-deficient rats (5, 28). A third sample was prepared by removing the tocopherols from a sample of carefully rendered pork back fat. The tocopherols were removed by dissolving 200 g. of the fat in 1000 ml. of low boiling petroleum ether and passing through two columns of activated alumina, 3 X 34 cm. (chromatographic alumina, Grade A, minus 80 mesh, Aluminum Ore Co., and Merck's Brockman Alumina). The solvent was then removed under vacuum. The three substrates all had induction periods of 10 minutes or less as measured by an oxygen absorption method at 100°C. They nevertheless appeared to contain a small amount of residual tocopherol and the optical densities obtained upon analysis were applied as corrections to the optical densities

TABLE I  
*Data Obtained for Reference Curve in Fig. 3*

Wt. of fat saponified	Content of added tocopherol	Initial transmission	Final transmission	Optical density (uncorrected)	Optical density* (corrected)
<i>a.</i>	<i>γ</i>	<i>per cent</i>	<i>per cent</i>		
Rat fat No. 1					
8.94	0.0	90.0	79.0	.057	—
8.96	26.9	94.0	64.0	.167	.139
8.85	53.1	95.0	45.0	.325	.297
8.61	51.7	92.5	40.0	.364	.337
8.69	52.1	94.5	39.0	.384	.356
8.78	79.0	95.0	25.7	.568	.540
8.65	77.9	95.2	26.0	.564	.536
Rat fat No. 2					
8.65	26.0	93.0	64.0	.162	.134
8.83	26.5	95.0	62.5	.182	.154
8.52	85.2	95.2	23.0	.617	.590
Hog fat					
8.37	0.0	92.0	89.0	.014	—
8.38	70.2	95.2	34.0	.447	.440
7.80	71.1	91.9	32.7	.449	.442

\* The corrected optical densities,  $D_c$ , were calculated using the formula

$$D_c = D_s - \frac{W_s}{W_b} D_b, \text{ where}$$

$D_s$  = measured optical density for the sample,

$D_b$  = measured optical density for the control (substrate)

$W_s$  = weight of sample

$W_b$  = weight of control.

found for the samples containing added tocopherol. The treated hog fat was particularly low in tocopherols and appears to be an ideal substrate for the measurements that are necessary in the preparation of a reference curve.

4. *Measurement of the Induction Periods of Rendered Hog Fats:* The stabilities of the fats were measured in a Warburg respirometer at 100°C. (28, 29). Following a period of slow and relatively constant rate of oxygen uptake, the absorption suddenly increased rapidly. The change in rate of absorption was sufficiently sharp to easily define the induction period within 10 minutes, which was the length of time between manometer readings.

5. *Other Measurements:* Official methods were used in measuring the iodine and thiocyanogen values (30), and the calculations of approximate compositions were based on empirical equations (31). Peroxide values were determined in some cases by a modified Wheeler method (32). Replicate measurements with good agreement were obtained in all analyses.

## RESULTS AND DISCUSSION

Referring to the calibration data in Table I, if the optical densities of the methylene blue reaction mixtures are corrected by the amounts of absorption obtained in blank determinations on the substrates, it is

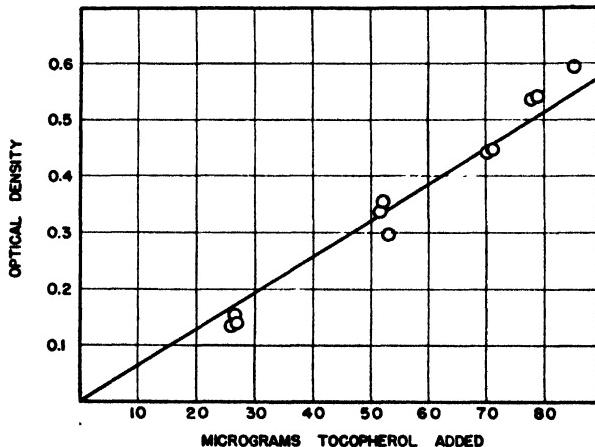


FIG. 3

Reference Curve Relating Optical Densities and Tocopherol Contents

found in Fig. 3 that the corrected optical densities and the added amounts of tocopherol are related within experimental error by a straight line passing through the origin. The average deviation of the corrected optical densities from the straight line is 9.2%.

For the three substrates used, this relationship is independent of the substrate within the limits of experimental error. The proportionality constant, K, is  $6.4 \times 10^{-3}$ . Since the K value obtained by Andrews and Binnington for a direct measurement of the optical densities of the orthoquinone solutions in comparable cuvettes is  $.225 \times 10^{-3}$ , an

TABLE II  
*Tocopherol contents of various hog fats*

Lard Sample	Weight saponified	Aliquot taken	Optical density	Tocopherol content per gram of fat ( $K = 6.4 \times 10^{-3}$ )	Average
1. Ruffle fat	8.92	{ 0.4 0.4	.610 .606	26.7 26.6	28.7
	8.84	0.4	.646	28.6	
	8.81	0.4	.696	30.9	
2. Leaf fat	9.24	{ 0.5 0.5	.707 .627	23.9 21.3	
	8.89	{ 0.4 0.4	.427 .421	18.8 18.5	
	8.59	0.4	.487	22.2	
	8.96	0.4	.486	21.4	
b. Hog No. 2					
3. Ruffle fat	9.26	1.0	.417	7.0	7.8
	9.23	1.0	.477	8.1	
	9.08	1.0	.479	8.2	
4. Leaf fat	8.85	1.0	.373	6.6	6.7
	8.75	1.0	.376	6.7	
	8.57	1.0	.367	6.7	
5. Ham facing	8.67	1.0	.292	5.3	5.3
	8.99	1.0	.289	5.0	
	9.11	1.0	.334	5.7	
6. Back fat	8.76	1.0	.371	6.6	6.4
	8.90	1.0	.356	6.3	

approximate 30-fold increase in sensitivity is obtained by introduction of the methylene blue modification.

The determinations of the tocopherol contents of several fats from two hogs are given in Table II. The average deviation of the individual values in column 5 from the average values in column 6 is 5.5%.

The hogs were selected at random and nothing is known concerning their history. The fats from the first hog were stored at -20°C. for one month before they were rendered. Even at this low temperature all of the cuts of fats except those from which samples 1 and 2 were obtained had developed appreciable peroxide values. The peroxide values of

TABLE III  
*Relation of Induction Periods to Fatty Acid Glyceride Compositions and Tocopherol Contents*

a. Hog No. 1					
Lard Sample	Saturated fatty acid glyceride	Oleic acid glyceride	Linoleic acid glyceride	Tocopherol content per gram of fat	Induction period
1. Ruffle fat	per cent 43.4	per cent 49.3	per cent 7.3	γ 28.7	min. 430
2. Leaf fat	41.7	47.4	10.9	21.3	270
b. Hog No. 2					
3. Ruffle fat	43.3	51.0	5.7	7.8	170
4. Leaf fat	39.0	51.6	9.4	6.7	115
5. Ham facing	31.3	58.6	10.1	5.3	88
6. Back fat	35.9	52.8	11.3	6.4	53

samples 1 and 2 were insignificant and further storage at -20°C. for several months after rendering did not result in any further measurable development of peroxides. All of the fats from the second hog, on the other hand, were rendered immediately. They showed no measurable peroxide content even after storage for several months at -20°C.

It is seen in Table II that fats from various parts of the same hog contain approximately the same amounts of tocopherol. In both hogs there was a slightly greater content in ruffle fat (mesentery) than in leaf fat. On the other hand, there was a striking difference between the two hogs in the amounts of tocopherol contained in their adipose tis-

sues. This difference is quite consistent with a suggestion that the hog, like the rat (5), derives its tocopherols solely from the diet.

Table III compares the various fats from the two hogs with respect to approximate glyceride composition, tocopherol content and induction period. Since the analytical method does not distinguish the three tocopherols, and since they have different antioxidant activities (33), some justification is necessary in making a comparison of the tocopherol contents in column 5 and the induction periods in column 6. If the tocopherols are derived from the diet,  $\beta$ -tocopherol, which is not abundant in plant fats, may be presumed to be almost absent.  $\gamma$ -Tocopherol, which is the most powerful as an antioxidant, is least readily absorbed and deposited in the adipose tissues of rats (34). If this is also true for hogs, it is probable that  $\alpha$ -tocopherol is preponderant. Comparing the two hogs, unless their diets were radically different, the relative proportions of  $\alpha$ -tocopherols in the total tocopherols of the adipose tissues were probably quite similar.

Unlike the tocopherol concentrations, the induction periods of the various fats in each hog were quite different. It is well known that linoleic acid in glycerides is more susceptible to attack by atmospheric oxygen than the less unsaturated acids. In the absence of more highly unsaturated acids, other things being approximately equal, the induction periods of fats are strongly influenced by the linoleic acid content. Comparing the data for samples 3, 4, 5 and 6 having only slight variations in tocopherol content, it is seen that the induction periods vary inversely with the linoleic acid contents. The differences in induction periods are, therefore, primarily due to differences in composition and only slightly to differences in tocopherol content. Comparing the same fats from different hogs, however, it is seen that the differences in induction periods are primarily due to differences in tocopherol content of the fats. Taking into account the magnitudes of these differences, the evidence is very strong that the inherent keeping qualities of carefully rendered fats from hogs are dependent primarily on two variables, their linoleic acid and tocopherol contents, and that tocopherols are the primary and possibly the only natural direct inhibitors of oxidation occurring normally in hog fats.

Conclusive proof of the latter point could probably be gained by measuring the induction periods of fats from grown hogs which had been raised on tocopherol-free diets. Facilities for such an experiment were not available, and an analogous experiment was therefore con-

ducted in this laboratory with rats. Proof was obtained that tocopherols are the only direct inhibitors of oxidation normally present in the adipose tissues of rats (5).

The concentrations of tocopherols in the fats of these two hogs are considerably higher than the concentration of 2 γ/gram reported by Karrer *et al.* for a single sample of hog fat (2). The fats from the first hog contain about the same concentration of tocopherol as is found in the fats from normal rats. There is a suggestion here that tocopherols may be as important biologically to hogs as to rats.

### SUMMARY

A method based on the Furter and Meyer reaction and developed by Binnington and Andrews for the determination of tocopherols in vegetable oils has been further modified to make possible the measurement of the small amounts of tocopherols in animal fats. The rather critical and detailed character of the procedure is more than offset by the high degree of specificity and sensitivity obtained.

The method has been applied to the measurement of tocopherols in hog fats. Evidence was obtained that tocopherol concentrations in several fats from the same hog are quite similar, but that the contents of the same fats in different hogs may vary widely. All of the values for several fats from two hogs were considerably greater than a single value previously reported in the chemical literature.

It was found that, in general, the keeping qualities of carefully processed hog fats are dependent primarily on two factors, their linoleic acid and tocopherol contents. It is concluded that tocopherols are the only natural direct inhibitors of oxidation present appreciably in normal hogs and that the tocopherols are probably derived solely from the diet.

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## LETTERS TO THE EDITORS

### Note on the Purification and Properties of Glutamic-Aspartic Transaminase

Some time ago we have presented data which suggested that a derivative of vitamin B<sub>6</sub> is involved in the enzymatic transamination of glutamic and aspartic acid (1). Controversial reports on this subject have appeared in the meantime. Leloir and Green (2) have purified the enzyme and stated that it does not contain a dissociable prosthetic group nor significant amounts of B-complex vitamins. On re-examination, however, the presence of a pyridoxine derivative became apparent (private communication from Dr. D. E. Green, Oct. 3, 1945). Cohen and Lichstein (3) could not find evidence for the role of vitamin B<sub>6</sub> in transamination.

We have purified the glutamic-aspartic transaminase from pig heart and found that the vitamin B<sub>6</sub> content is greatly increased with rising activity. In contrast to earlier reports (4) we found that purified transaminase is a very stable enzyme. It is readily obtained in quantity by the following procedure:

Ground pig heart (Q<sub>T</sub> 800–1000) is dehydrated with an excess of acetone, pressed and dried. The resulting product retains its activity for many months and is a convenient source material. The dry powder is extracted with 10 vol. of 0.04 M Na<sub>2</sub>HPO<sub>4</sub> at 60°C. for 30 minutes (Q<sub>T</sub> 1500). After filtration inert protein is removed by acidifying to pH 5.0; the filtrate (Q<sub>T</sub> 3000) is neutralized and fractionated with sat. (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-NH<sub>4</sub>OH solution of pH 8.0. The enzyme is found in the fractions between 55 and 70% saturation. The precipitate (Q<sub>T</sub> 5000–10,000) is dissolved in 0.1 M phosphate buffer of pH 7.5 and dialyzed using first phosphate buffer, then dist. water. The pH of the solution is adjusted to 5.8 and alumina Cγ suspension (20 mg. per ml.) is added in portions. The first fractions (< 0.2 vol. alumina) contain little active material. All enzyme is adsorbed when 0.6 vol. of alumina is added. After washing, the enzyme is eluted with 0.1 M phosphate buffer (Q<sub>T</sub> ~ 25,000). The presence of a pyridoxine derivative (more than 500γ per g. in our best preparations) is readily discovered by

microbiological assays or by chemical methods. The glutamic acid-alanine transaminase is separated by this purification. The prosthetic group is rather firmly bound in glutamic-aspartic transaminase. In solution the enzyme is stable for months, but it is very sensitive to sunlight which causes a loss of activity corresponding to the destruction of vitamin B<sub>6</sub> compounds (5).

The nature and mode of action of the prosthetic groups are under investigation.

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# **Human Biotin Metabolism on Various Levels of Biotin Intake \***

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Received August 7, 1945

## **INTRODUCTION**

The importance of biotin in the nutrition of man and animals has been well established. The animal work on biotin and egg white injury, the isolation and characterization of biotin and avidin, and the requirement of microorganisms for biotin have been reviewed (1, 2, 3, 4, 5).

In the present study the urinary and fecal biotin elimination has been determined on diets of low, moderate and high biotin content and a moderate biotin diet with the daily administration of 50 and 100  $\gamma$  of biotin as the methyl ester (70% purity).† The diurnal variation in excretion of biotin of certain subjects consuming these diets is presented. The water-soluble biotin in feces has also been measured.

## **EXPERIMENTAL**

*Subjects:* In the two studies reported, uniform weighed diets were eaten by 10 healthy young women. Six subjects ate the three diets varying in biotin content and four were in the second study. One subject began the first period of study on the fifth day of the experiment. The weight of each subject varied little during the course of the study.

*Diets:* The composition of the diets is given in Table I. The first study lasted 30 days and was divided into three consecutive 10 day periods. The three diets of low, moder-

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\* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Nutrition Foundation Inc., New York.

† The authors wish to express their great appreciation to Dr Y. Subbarow of Lederle Laboratories, Inc., for the generous supply of biotin used during these experiments.

TABLE I  
*Composition of Diets*

Food	Study 1		
	Low biotin, diet 1	Moderate biotin, diet 2*	High biotin, diet 3
orange juice, canned	150	—	200
grapefruit juice, canned	—	200	—
bread, white	65	43	—
bread, whole wheat	—	—	21
butter	20	15	10
jelly, grape	20	15	8
all bran, Kellogg's	—	—	50
egg	—	—	50
milk	—	—	300
Biotin content of breakfast	2γ	2γ	39γ
<i>Lunch:</i>			
baked beans, canned	150	—	—
bread, white	43	—	—
butter	15	12	8
lettuce	50	60	60
mayonnaise	15	15	10
cheese, cheddar	30	55	—
eggs, scrambled	—	—	150
bread, whole wheat	—	60	21
apple	150	150	—
ice cream	—	—	100
peanuts	—	—	20
milk	—	240	260
Biotin content of lunch	2γ	12γ	43γ
<i>Dinner:</i>			
cottage cheese	100	—	—
potato, baked	150	100	100
beef, ground round	—	100	—
liver, beef	—	—	100
beans, green snap	—	—	100
tomato juice, canned	—	200	200
lettuce	10	—	—
corn, canned	100	—	—
cabbage	—	30	—
bread, white	18	23	—
butter	25	23	10
ice cream	—	100	—
grapefruit sauce, canned	100	—	—
apricots, canned	—	—	100
milk	—	240	240
Biotin content of dinner	4γ	19γ	89γ

\* This diet was also used in study 2.

ate and high biotin content contained by analysis 9, 33 and 171  $\gamma$  of biotin daily. The water intake was not kept constant.

The diet low in biotin was deficient in several essentials. Therefore, it was supplemented daily with 1 mg. of thiamine, 1 mg. of riboflavin, 15 mg. nicotinamide, and 0.65 g. of calcium lactate. The low-biotin diet contained no milk or meat in order to keep the biotin content low.

To the diet with a moderate level of biotin, 1 mg. of thiamine was added with lunch. This diet was very similar to that described by Parsons and Collord (6). This moderate-biotin diet contained a pint of milk and a serving of ground beef.

TABLE II  
*Nutritive Value of Diets*

Dietary essential	Low-biotin, diet 1	Moderate-biotin, diet 2	High-biotin, diet 3
Calories	1859	2080	2198
Carbohydrate, g.	207	182	164
Fat, g.	82	114	118
Protein, g.	62	75	108
Calcium, g.	0.56	1.34	1.45
Phosphorus, g.	1.37	1.45	2.54
Iron, mg.	8.1	10.2	32.2
Vitamin A,* mg.	1.5	1.3	9.6
Ascorbic acid, mg.	161	181	236
Thiamine, mg.	2.1	1.1	2.3
Riboflavin, mg.	2.2	2.4	6.9
Pantothenic acid, mg.	2.1	4.0	18.3
Nicotinic acid, mg.	18.9	12.0	50.5
Biotin, $\gamma$	8.9	32.8	171.0

\* Carotene was expressed in terms of vitamin A on the basis that 0.6  $\gamma$  of carotene are equivalent to 0.3  $\gamma$  of vitamin A.

The high-biotin diet included four eggs, beef liver and nearly a quart of milk daily. These foods are particularly rich in biotin. The levels of the other vitamins as well as the amount and quality of protein were increased over the low and moderate-biotin diets (Table II).

The basal dietary constituents in the second study were the same as in diet 2 of the first study. During periods II and III, 50 and 100  $\gamma$  of biotin as the methyl ester (70% pure) were added in two equal doses before 9 A.M. with breakfast and at 6 P.M. with dinner.

**Methods:** An aliquot of the foods eaten by the subjects on each diet was treated as previously (7) except that 4 N HCl was used instead of 2 N H<sub>2</sub>SO<sub>4</sub>, since the former was proved to yield a greater amount of biotin. Twenty-four hour urinary collections were made as in the foregoing studies. During diets 1, 2 and 3 (study 1) the first two

days' collections were discarded. The urinary and fecal collections for days 3 through 6 and 7 through 10 were composited so that a representative sample would be obtained. In the second study the biotin content of each 24-hour urinary sample was assayed.

Diurnal variation in biotin content of the urine was studied for the last day of each period (tenth day of study 1 and the sixth day of periods II and III of study 2). Aliquots of the collections for each period were assayed for biotin for subjects E. B. and J. G. on diets 1, 2 and 3 and for all four subjects during study 2 with the exception of V. S. during period II.

Fecal eliminations were collected, dried and hydrolyzed as described previously except that 4 N HCl was used instead of 2 N H<sub>2</sub>SO<sub>4</sub>. In diets 1, 2 and 3 (study 1) the feces were composited as were the urinary collections, days 3-6 and days 7-10 together. In study 2, period I the eliminations for days 1-4 and 5-6 were combined and thereafter days 1-3 and 4-6 were combined. In all cases 0.5 g. of carmine in a gelatine capsule was ingested to mark the beginning and end of each period.

The amounts of biotin extracted from wet and dried samples of a single fecal elimination by water and by 4 N HCl in 4 hours at room temperature were compared. Chloroform (1 ml.) and 50 ml. of water were added to 5 g. of wet feces. A portion of the water extract was hydrolyzed in the usual manner with 4 N HCl as well. A second aliquot of this fecal sample was dried and portions of it extracted with water and hydrolyzed with acid.

## RESULTS AND DISCUSSION

### *Metabolism of Biotin in Foods*

The urinary biotin of the six subjects did not show a consistent change when the dietary biotin was increased from 9 to 33  $\gamma$  per diem, an increase of 370% in biotin intake (Table III, diets 1 and 2). In fact, the average urinary biotin for five subjects was almost identical for these two diets (29 and 30  $\gamma$ ). The biotin excretion for five of the six subjects fell within a narrow range and that of the sixth was conspicuously lower than the others during all three levels of biotin intake (Table III). Subject E. W. P. was known to have eaten, previous to this study, less than three meals a day which probably entailed an abnormal selection of food. Possibly some of the biotin of the moderate and high-biotin diets was retained to elevate low body stores of this vitamin. Another possibility would be less intestinal synthesis than the other subjects. No evidence for this was afforded by the level of her fecal biotin, however, as it was very similar to the average of the other subjects. Unfortunately we were unable to study her response to the ingestion of pure biotin.

All six subjects excreted slightly less biotin on days 7-10 during the low and moderate levels of biotin intake than on days 3-6. It is possible,

though not probable, that the urinary biotin would have fallen to the level of intake had the period been extended longer than the 10 days. Oppel (8) found that during an unrestricted diet or after a period of starvation similar amounts of biotin were present in human urine. These observations suggested to him that urinary biotin was based on a relatively constant level of biotin synthesis by colonic bacteria and sudden variations due to changes in biotin content of the diet.

TABLE III  
*Urinary and Fecal Biotin During Three Levels of Biotin Intake  
(Diets 1, 2 and 3, study 1)*

Diet	Days	Daily biotin intake	Daily urinary biotin			Daily fecal biotin		
			5 subjects		Subject E.W.P.	5 subjects		Subject E.W.P.
			Range	Av.		Range	Av.	
1	3-6	γ	γ	γ	γ	γ	γ	γ
	7-10	9	24-38	31	25	51-84	62*	52
	Av.	9	20-36	27	18	41-75	55	63
2	3-6	33	29-35	32	17	63-108	86	82
	7-10	33	22-30	27	12	46-99	73	77
	Av.	33	27-32	30	15	61-88	78	80
3	3-6	171	123-172	150	94	75-143	105	108
	7-10	171	168-200	185	108	64-147	96	103
	Av.	171	157-180	167	101	71-132	100	105

\* Average of only four subjects.

When a high-biotin diet (171 γ daily) was consumed, the urinary biotin reflected the rise in intake (Table III). This rise is in contrast to the lack of change in urinary biotin from low to moderate intake. The dietary intake was raised 520% while the average urinary output of biotin for five subjects increased 500% (days 3-6) and 620% (days 7-10) over the average of the preceding periods. Thus, the urinary excretion of biotin was distinctly higher during the last four days of the high-biotin diet. A plateau of high biotin excretion on this high-biotin diet was not reached in 3-6 days. There was a tendency for the body

to retain quite large amounts of biotin, thus making a lag in reaching a plateau of urinary biotin excretion.

The fecal elimination of biotin on the three levels of biotin intake is shown in Table III. Possible irregularities in daily elimination of feces were somewhat minimized by pooling 4 days' samples as described previously.

The significance of differences in fecal biotin is very difficult to ascertain. Variations in fecal biotin due to dietary biotin may be masked by variations in biotin synthesized by bacteria. The food residues are an important factor in determining the growth of the intestinal bacteria and consequently their synthesis of various compounds. There is no way of distinguishing between biotin of food and microbial origin.

The fecal biotin exceeded the biotin in the diet on the low and moderate-biotin diets (Study 1), but did not equal the intake on the high-biotin diet. Biotin synthesis in the feces was more obvious when biotin intake was relatively low. The ratio of fecal biotin to dietary biotin for the low, moderate and high-biotin diets was as follows (average for six subjects): 6 : 1, 2.4 : 1 and 0.6 : 1. There was, however, an absolute increase in fecal biotin from the low to moderate to high-biotin diets in a ratio of about 1 : 1.4 : 1.8 (Table III), although there was considerable individual variation. The increase in fecal biotin was nearly accounted for by the increase in weight of feces (1 : 1.2 : 1.8, for diets 1, 2, and 3). In individual cases the biotin per gram of feces did not show a direct relationship to the amount of feces eliminated. The greater weight of feces on diets 2 and 3 was not due entirely to differences in fiber content of the diet but may possibly have been due to more bacterial cells or residues.

Thus, in the three diets used, the fecal biotin increased to a limited extent when markedly different amounts of biotin in foods were ingested. Oppel (8) found no suggestion of a correlation between dietary biotin and fecal biotin on the diets he employed, but he did suggest the existence of a correlation between urinary and fecal biotin. The data from the present study do not entirely substantiate this latter suggestion in that during the moderate-biotin diet the fecal biotin was increased over the low-biotin diet while the urinary biotin was not.

The division of biotin output between urine and feces is of interest. The ratio between urinary and fecal biotin averaged 1 : 2, 1 : 2.6 and 1 : 0.6 for the low, moderate and high-biotin diets, respectively.

*Metabolism with Additions of Pure Biotin (Study 2)*

The daily urinary excretions of biotin by four subjects on a moderate-biotin intake and on the same diet supplemented with biotin methyl ester equivalent to 50 and 100  $\gamma$  of free biotin are shown in Table IV.

TABLE IV

*Urinary and Fecal Biotin During a Moderate-Biotin Diet and with Added Pure Biotin (Study 2)*

Period and day	Biotin intake		Urinary biotin		Fecal biotin	
	Food	Added biotin	Range	Av.	Range	Av.
I*	35	0	29-42	34	γ	γ
	35	0	26-36	30	40-100	67
	35	0	27-46	37		
	36	0	25-35	32	37-148	87
II	35	50	30-41	36		
	35	50	42-54	46		
	35	50	38-56	43	23-83	54
	35	50	46-52	49		
	35	50	35-45	39		
	35	50	40-53	45	53-70	55
III	35	100	56-71	62		
	35	100	51-71	61		
	35	100	68-88	79	28-72	54
	35	100	69-86	79		
	35	100	58-111	91		
	35	100	88-117	106	40-90	70

\* Samples for days 1 and 2 were not assayed as they represent a transition period from the unrestricted diet.

Two of the four subjects were the same as those on diets 1, 2, and 3 of the first study.

When 50  $\gamma$  of biotin were ingested in two equal doses with breakfast and dinner, the urinary biotin showed a slight rise the first day of the addition in the case of two of four subjects (period II, Table IV). An

increased urinary biotin was observed for all subjects on the second day; a maximum occurred on the fourth day for three of the four subjects; on the fifth and sixth days there was a leveling off of biotin excretion. When an added 100  $\gamma$  of biotin was ingested (period III, Table IV), the urinary biotin of all subjects rose the first day and in general continued to rise for the entire six days of the experiment. A larger percentage of added biotin was found in the urine with the higher supplement than with the lower.

In period I the urinary biotin equaled the intake. When 50  $\gamma$  of biotin were added, an average of 50% of the total intake was excreted and 70% when 100  $\gamma$  of biotin were added (average of days 4-6, Table IV). Increased biotin intake supplied by synthetic biotin and beef liver caused similar increases in urinary biotin, but raw or boiled bakers' yeast did not cause so marked an increase (7).

The biotin content of the feces during the second study is recorded in Table IV. As in the former studies the daily variation in biotin elimination was great for the four subjects. The daily average for the three periods was 77, 55 and 62  $\gamma$ , respectively, for the moderate-diet (period I), moderate plus 50  $\gamma$  of biotin and the moderate plus 100  $\gamma$  (periods II and III). Thus, the ingestion of 50 or 100  $\gamma$  of biotin as the methyl ester did not increase the fecal biotin over that during the first period. The added biotin unaccounted for in the urine then, must have been stored, destroyed or absorbed by the intestinal flora. The microorganisms may have synthesized less biotin when a supply of unbound biotin was present.

The total output of biotin during the moderate-biotin diet averaged 3.4, 1.2 and 1.2 times the biotin intake during the three periods. As in diets 1, 2 and 3 (study 1) the greatest excess output over intake occurred during the lowest biotin intake.

The water-soluble biotin in dried feces eliminated during days 4-6 of the second study was determined. If there were no changes in drying, this water-soluble biotin would presumably represent biotin available for absorption, but not absorbed. Only 2-15% of the total fecal biotin was water-soluble (2-9  $\gamma$  daily). There was slightly less water-soluble fecal biotin during the moderate-biotin diet (period I) than during the periods when 50 and 100  $\gamma$  of added biotin were ingested. The average for these three periods was 4, 12 and 8% or 3, 7 and 6  $\gamma$  of water-soluble biotin daily. It is entirely possible that a small part of the added biotin was excreted unchanged, which might account for the

differences in the water-soluble biotin in the feces during the three periods.

A further study of water-soluble biotin in one sample was made immediately after elimination and also after drying. The water-soluble biotin increased after drying from 3 to 9% (5 to 14 $\gamma$ ). When the two water extracts of feces were hydrolyzed with acid, the measurable biotin was increased to 6 and 12% (10 and 20 $\gamma$ ) for the moist and dried feces. Acid hydrolysis of moist or dried feces resulted in nearly equal extraction of biotin (162 $\gamma$ ). Which fractions of biotin are available for human use are not known and the problems of form and availability need further study.

The diurnal variation in urinary biotin of certain subjects on the last days of all diets was determined. The lowest hourly excretion of biotin occurred in the hours during sleep (12 P.M. to 8 A.M.), an average of 3% per hour. The excretion during the morning (8 A.M. to 12 M.) was only slightly higher than the hours during sleep (average of 3.5% per hour). The greatest excretion came in the afternoon from 12 M.-6 P. M. (5% per hour) and in the evening from 6 P.M.-12 P. M. (5% per hour). Nearly 2/3 of the biotin was excreted in the afternoon and evening (12 M.-12 P.M.).

Biotin methyl ester when administered orally in amounts of 25 or 50 $\gamma$  with breakfast and with dinner was excreted rather rapidly. A large portion of the biotin appeared in the urine in the first six hours after ingestion: the afternoon and evening excretions were high in biotin. However, the added biotin was not completely excreted within six hours after ingestion as indicated by the fact that biotin excretion for the hours during sleep was higher when the 25 or 50 $\gamma$  of biotin was added than during the moderate-biotin diet with no biotin addition (period I, Table IV).

#### SUMMARY

Two studies of the biotin metabolism of 10 college women who consumed a low, moderate and high-biotin diet and the moderate diet supplemented with 50 and 100 $\gamma$  of biotin as the methyl ester are presented. These diets contained from 9 to 171 $\gamma$  of biotin daily.

Increasing the biotin intake in food from 9 to 33 $\gamma$  failed to cause a rise in urinary biotin, but the fecal biotin increased 40%. When 171 $\gamma$  of dietary biotin were ingested, the urinary biotin rose markedly to

more than 5 times that excreted on the low or moderate biotin diet, while the fecal biotin increased 80% over that of the low-biotin diet.

Total biotin output averaged 9, 3 and 1.5 times the dietary intake for the low, moderate and high-biotin diets, respectively. Thus, biotin synthesis was again indicated and relatively less synthesis seemed to take place during the high-biotin diet.

When 50  $\gamma$  of biotin were added to the moderate-biotin diet, a maximum of 30% of the additional biotin was accounted for in the urine. The maximum urinary output occurred on the third or fourth day after the beginning of the biotin feeding.

A larger percentage of biotin was recovered when 100  $\gamma$  of biotin were ingested. A maximum of 72% was accounted for in the urine. The rise in excretion continued for the entire six days of higher intake.

Fecal biotin did not rise when either 50 or 100  $\gamma$  of biotin were fed. The water-soluble biotin in dried feces varied from 2–15% of the fecal biotin. An increase in measurable biotin occurred when a water extract of feces was acid hydrolyzed.

The diurnal variation in urinary biotin was studied in diets of low, moderate and high-biotin intake. The hourly biotin excretion was greatest in the afternoon and evening and lowest during sleep and in the morning. Twenty-five or 50  $\gamma$  of biotin given with breakfast and dinner were excreted promptly, a large portion but not all of the additional biotin being found in the urine within six hours after ingestion.

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## Liver Arginase

### I. Preparation of Extracts of High Potency, Chemical Properties, Activation-Inhibition, and pH-Activity

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Received August 22, 1945

#### INTRODUCTION

The enzyme arginase was discovered in 1904 by Kossel and Dakin (1). It normally decomposes *l*-(+)-arginine into ornithine and urea. This reaction, in the Krebs and Henseleit cycle (2), is part of the mechanism by which the nitrogen derived from the catabolism of the amino acids is converted into urea in the mammalian liver. A similar cycle of nitrogen transformation has recently been observed in the bread mold, *Neurospora crassa* (3). Arginase has been found to occur only in the livers of animals having a ureotelic metabolism (4, 5). Decomposition of arginine by liver arginase has been developed into a specific method for the estimation of this amino acid (6, 7). An intriguing problem in the chemistry of arginase is the mode of its activation by certain divalent metallic cations, *e.g.*, Mn<sup>++</sup> and Co<sup>++</sup> (8, 9).

The biological importance of this enzyme led the authors to undertake the investigations reported here.

#### MATERIALS AND EXPERIMENTAL METHODS

*Arginase Solutions.* In the course of the experimental work, arginase extracts were prepared from beef, dog, rat and rabbit livers. Solutions of arginase were, unless otherwise stated, put up and stored in 0.1 *M* phosphate buffer solution of pH 7.0. They were diluted to the desired degree to test the enzyme activity. Activators were used only when so specified.

\* This article is derived from a thesis submitted to the Graduate Division of the University of California by Mostafa Safwat Mohamed in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Biochemistry, October, 1943.

*Arginine Solutions.* Solutions of the desired concentration were prepared from a 0.2 M stock solution of *l*-(+)-arginine hydrochloride, adjusted to pH 7.0 with sodium hydroxide.

*Measurement of Arginase Activity.* The enzymatic activity of each of the different arginase preparations were determined from the amount of urea formed. The urea was estimated by the xanthydrol-urea method of Allen and Luck (10). The advantage of this method is that the visual observation of the amount of dianthydrol-urea precipitate provides an immediate qualitative test of the variations in the degree of arginase activity. Such a test is useful in deciding on the effectiveness of different preparative procedures and on the effect of various activating and inhibiting agents.

In the usual procedure employed in testing for arginase activity, 0.1 ml. aliquots of suitably diluted arginase solutions were allowed to act for 15 or more minutes on 0.2 ml. portions of 0.2 M arginine solution (adjusted to pH 7.0) mixed with 7.7 ml. of a selected buffer. At the end of the incubation period, 2 ml. volumes of 20% acetic acid were added to stop the reaction. To determine the amount of urea formed, 1 ml. aliquot of the test solution was pipetted into a 15 ml. conical centrifuge tube and to this there were added 1 ml. of glacial acetic acid and 0.1 ml. of a saturated solution of xanthydrol in absolute methanol. After standing for one hour, the dianthydrol-urea precipitate was isolated by centrifugation and the quantity of this derivative was determined by oxidation with a sulfuric acid solution of potassium dichromate.

*Units of Arginase Activity.* For ordinary comparative purposes, the arginase activity was expressed as the mg. of urea produced in the 15 or 30 minutes of incubation. For intercomparison among different preparations, and under widely varying experimental conditions, the arginase activity was expressed in terms of a unit similar to the one formulated by Richards and Hellerman (11). Under the same conditions of temperature and pH it is 2.3 as large as Richards and Hellerman's unit because natural instead of common logarithms are employed in the first order reaction equation.

These authors observed that the arginase reaction could be represented by the equation for a first order reaction over a range between 10 and 75% hydrolysis at pH 7.5. The reaction constant under these conditions is directly proportional to the enzyme concentration. In kinetic studies, reported in another paper (12), we have verified the observation that the arginase reaction fits a first order equation for a large part of its course. These considerations led us to formulate the following units of arginase activity (*AU*) and of enzyme purity (*AP*).

$$k = \frac{1}{t} \left( 2.3 \log \frac{\text{potential urea (mg.)}}{\text{potential urea (mg.)} - \text{urea formed (mg.)}} \right) \quad (1)$$

where the time, *t*, is expressed in hours.

$$AU = \frac{k}{\text{sample volume}} \quad (2)^1$$

$$AP = \frac{AU \text{ per ml.}}{N \text{ of enzyme preparation (mg. per ml.)}} \quad (3)$$

<sup>1</sup> *AU* are usually expressed per ml. of solution but may be expressed as the content of the total extract.

The nitrogen content of the enzyme preparations was determined by the micro-Kjeldahl method.

In the same arginase preparation the unit value will of course vary unless the temperature, pH of the reaction mixture, and the degree of activation of the enzyme by metallic ions are maintained constant. The values of temperature and pH selected were 40°C. and 8.5 respectively; maintenance of a constant degree of activation, however, could not be assured.

### PREPARATION OF ARGINASE OF HIGH POTENCY

Preliminary to the study of the properties of arginase, much attention was devoted to developing a comparatively simple procedure for isolating potent preparations of the enzyme. The essential steps in the procedure finally adopted as being most convenient and successful are: (1) extraction of the arginase from fresh minced liver with 5% sodium acetate solution containing 5 mg.  $Mn^{++}$  per ml.; and (2) the preferential denaturation and precipitation of non-arginase protein

TABLE I  
*Arginase Activity at Various Stages of Purification*

Extract*	Volume ml.	AU† per ml.	Total AU†	Total N mg. per ml.	$AP^{\dagger} = AU/N$
A	6,000	126.0	756,000	6.0	21.0
B	9,500	82.5	784,750	1.3	60.9
C‡	12,630	60.2	760,300	—	—
D	1,200	625	750,000	2.2	284
E	240	2962	710,000	6.5	455.6

\* Letters refer to the stage of purification given in diagram.

† For definition of the terms  $AU$  and  $AP$ , see equations (1) to (3). Activity determinations carried out at 40°C. and pH 8.5 for 30 minutes.

‡ Dialyzed against distilled water for 8 days and then activated with  $Mn^{++}$ .

first by lead acetate and then by acetone. An outline of the steps involved in such an isolation is shown in the accompanying diagram. The arginase activities and nitrogen contents of the different fractions were tested at each stage of the purification. The results of these tests are recorded in Table I. It will be noted that the isolation method used is quite sparing of the enzyme, the loss in activity in the final step amounting to only about 6% of the enzyme content in the initial extract.

Because of the superiority of sodium acetate for the extraction of arginase and of this salt and of  $Mn^{++}$  for maintaining the stability of the enzyme, they were selected as extractives. A summary of experiments which demonstrates this is given in Table II.

TABLE II  
*Extraction of Arginase by Various Solvents*

Fresh liver extracted with	Total nitrogen day of extraction	Arginase activity			
		Day of extraction	4th day	6th day	10th day
<i>Mg. urea produced in 30 min. at 20°C. and pH 9.55</i>					
1) Distilled water	3.55	0.50	0.46	0.38	0.26
2) 2% NaCl	—	0.34	0.32	—	—
3) 0.05% NH <sub>4</sub> OH	4.65	0.20	0.07	0.03	0.0
4) 5% CH <sub>3</sub> COONa	4.00	0.56	0.59	0.66	0.66
5) 0.05 M Na <sub>4</sub> P <sub>2</sub> O <sub>7</sub>	4.55	0.44	0.50	0.59	—
6) 1% CH <sub>3</sub> COOH	4.00	0.08	0.04	0.18	0.0
7) MnSO <sub>4</sub> solution (5 mg. Mn <sup>++</sup> per ml.)	3.05	1.04	1.09	1.24	1.24

Arginase is quite stable in the presence of high concentrations of lead ions. The lesser stability of other proteins of the liver to lead ions, makes it an effective agent in the purification of arginase. Experiments which demonstrate this point are recorded in Table III.

The enrichment in enzyme activity by the method of isolation

TABLE III

*Effect of Varying Concentrations of Lead Acetate on the Activity of Beef Liver Arginase*

Five ml. portions of sodium acetate-extracted beef liver were treated with calculated volumes of 0.024 M Pb(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>·3H<sub>2</sub>O solution; each tube was allowed to stand for 30 min. at 20°C. and then centrifuged for 15 min. to remove the coagulum. Activity determinations were carried out at 40°C. and pH 8.5 for 30 minutes.

Per cent lead acetate solution in total volume of mixture	Total AU	Nitrogen content	= AP	Total AU	Nitrogen content	= AP
			AU/N			AU/N
		<i>mg. per ml.</i>			<i>mg. per ml.</i>	
0	209	5.5	7.6	258	5.6	9.2
20	250	4.3	9.3	226	3.7	11.3
33.3	—	—	—	323	2.8	15.0
50	274	2.3	11.7	368	2.0	18.1
60	196	1.4	11.0	363	1.6	18.6
66.7	185	1.1	11.6	425	1.3	22.6
75	165	0.7	11.7	228	0.7	16.2

described here is over 20 fold. Preparations of as high a degree of purity have been obtained, apparently, only by Richards and Hellerman (11) and by Van Slyke and Archibald (13). The last named authors, in a preliminary communication, reported obtaining arginase in which, on the basis of electrophoresis measurements, the enzyme comprised 35% of the total preparation.

*Properties of Purified Arginase.* Our most highly purified arginase preparations were grey when precipitated, but in solution they were colored a greenish brown comparable to the color of olive oil.

Tests in the Tiselius electrophoresis apparatus showed that the best of the arginase preparations were not homogeneous, there being either 3 or 4 moving boundaries. The major part of the protein was divided between two of the moving boundaries in about equal concentration. The electrophoresis runs<sup>2</sup> were carried out at the pH values of 7 and 5. Plots of the mobilities against pH yielded the values of pH 5.13 and 4.90 respectively, for the isoelectric points of the two chief protein components. Determination of isoelectric point, by titrating buffered solutions of the arginase with acetone to development of a permanent turbidity, yielded a value of pH 5.0. The value of pH 5.0, for the isoelectric point of arginase has been reported by Rossi (14) but the active component in the arginase preparation of Van Slyke and Archibald had an isoelectric point of pH 6.6.

In addition to arginase, it was found that the purified preparations were high in catalase activity. No effort was made to determine whether other enzymes were also present.

Arginase is quite stable at refrigerator temperatures, even when kept in solution. An arginase solution from beef liver lost only 8% of its activity over a period of 8 months. Left standing at 25°C., there was no detectable loss in 8 days.

*pH Activity Curves.* It appeared desirable to us to study the pH activity curves of purified arginase. A series of such curves for arginase from beef liver, activated in a number of different ways, are shown in Fig. 1. The arginase activity is given in terms of the amount of urea liberated in 30 minutes at the temperature of 40°C. The pH values were checked initially and at the end of each run by means of a glass electrode.

<sup>2</sup> We are greatly indebted to Dr. Harold P. Lundgren and Dr. W. H. Ward of the Western Regional Laboratory, U. S. Department of Agriculture, Albany, California, for their generosity in performing the electrophoretic analysis.

Our curves resemble the ones published by Hunter and Morrell (15) rather than those published by Hellerman and Stock (16). Fig. 1 shows that the form of the pH-activity curve is essentially the same for untreated and  $Mn^{++}$  activated arginase.  $Co^{++}$  and  $Ni^{++}$  activated arginase shows the dip in activity at pH 7 to 8 observed by Hunter

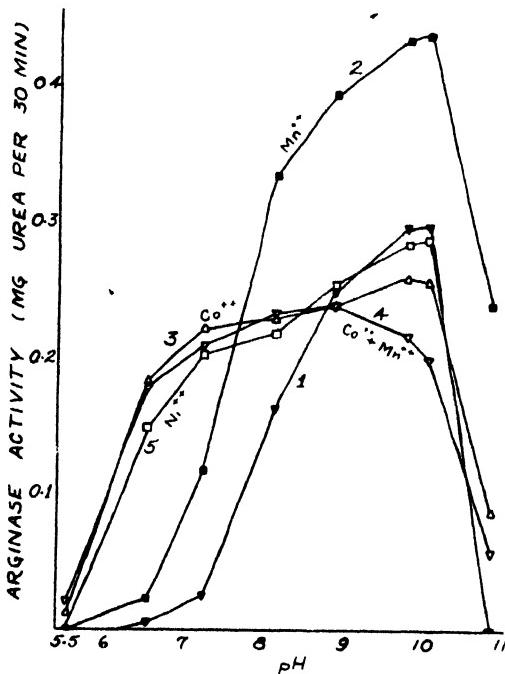


FIG. 1

#### Influence of Activating Cations on pH Activity Curves of Beef Liver Arginase

Curve 1 represents purified beef arginase dialyzed against distilled water. Remaining curves are for this enzyme preparation incubated with the indicated metallic ions for a period of 90 minutes at 40°C. before being adjusted to the desired pH and tested for arginase activity. The buffers employed were sodium veronal for the pH range 4 to 9.75, glycine for pH 10.0 and phosphate for pH 10.8.

and Morrell. All the pH-activity curves are decidedly asymmetrical with the highest activity being observed at pH 10.0 for all but arginase activated by a mixture of  $Mn^{++}$  and  $Co^{++}$ . In this case, the optimum activity was shifted to the more acid value of pH 8.5 to 9.0.

The pH activity curves of the liver arginase from a number of other

mammalian species are shown in Fig. 2. This figure shows that the curves for dog, rabbit, and rat liver arginase, under similar conditions, closely resemble the pH-activity curve of beef liver arginase. Fig. 2

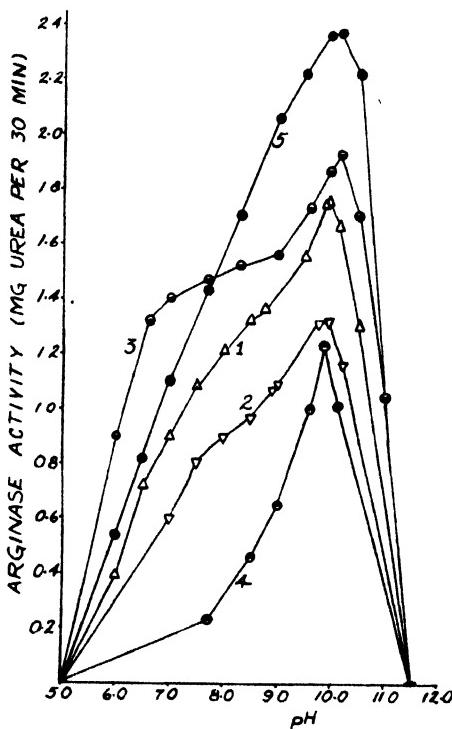


Fig. 2  
pH Activity Curves of Liver Arginase from Different Animal Species  
and the Effect of Borate Thereon

Curve 1, rat arginase. Curve 2, rat arginase in borate buffers. Curve 3, cobalt-activated dog arginase. Curve 4, cobalt-activated dog arginase in borate buffers. Curve 5, manganese activated rabbit arginase. Except in cases where borate was employed, the buffers used were phthalate (pH 4 to 5), acetate (pH 4 to 5.5), orthophosphate (pH 5.7 to 8.0), pyrophosphate (pH 7.9 to 9.2), and glycine (pH 8.3 to 12) at a final concentration of 0.1 M.

shows a phenomenon that will be discussed below; namely, the inhibiting effect of borate on arginase activity. A similar inhibition was observed with citrate. It will be noted from curves 2 and 4 that the

inhibition of arginase by borate extends over the whole pH range over which the enzyme is active.

An effect was made to interpret the pH-activity curve of arginase on the basis of the theory of enzyme kinetics formulated by Michaelis and Menten (17), assuming that one particular ionic form of the enzyme and of the substrate combined to form the active enzyme-substrate intermediate that initiated the reaction. According to this viewpoint, the reaction velocity would be proportional to the product of the concentrations of the active forms of enzymes and substrate. These concentrations, in turn, would vary with pH in accordance with the acid dissociation curves of the chemical species involved. From examination of the titration curve of arginine, Hunter and Morrell suggested that it is possible, though hardly certain, that the monovalent cation is the only form of arginine susceptible to attack by the enzyme. The only possible alternative is that it is the dipolar ion of arginine that reacts to form the active enzyme-substrate intermediate. Similar reasoning leads to the conclusion that the active form of the enzyme is either in the isoelectric or anionic state. We favor the view that the active form of arginine is the monovalent cation and the active form of arginase is anionic.

Employing the pertinent dissociation constant of arginine ( $pK = 9.04$  (18)) the dissociation constant of the active form of arginase was calculated to have the approximate value of  $pK = 7$ .

#### ACTIVATION BY METALLIC IONS

The activation of arginase by the divalent cations of manganese, cobalt and nickel has intrigued many investigators. However, no satisfactory explanation has been offered which adequately accounts for the activation of this enzyme by metal ions. In the present investigation further experiments were undertaken on the conditions for activation.

It was readily demonstrated that activation depends upon a measurable reaction between enzyme and ion activator and not between substrate and ion. Incubating buffered solutions of arginine with  $Mn^{++}$  and then adding enzyme, does not increase the velocity of splitting of arginine over the rate obtained when all the components are mixed immediately.

On the other hand, incubation of arginase with  $Mn^{++}$  or  $Co^{++}$  leads

to an increase in activity, which eventually reaches a maximum value. This effect is shown by the curves in Fig. 3.

It will be noted that the enzyme activity reaches the same final value with either manganous or cobaltous ion. Cobalt has a greater accelerating effect on the activation of arginase than does manganese. The reaction is greatly increased by raising the temperature. The maximum activation was reached in 3 hours incubation at 40°C, while 48 hours incubation was required at 20°C. with the same amount of

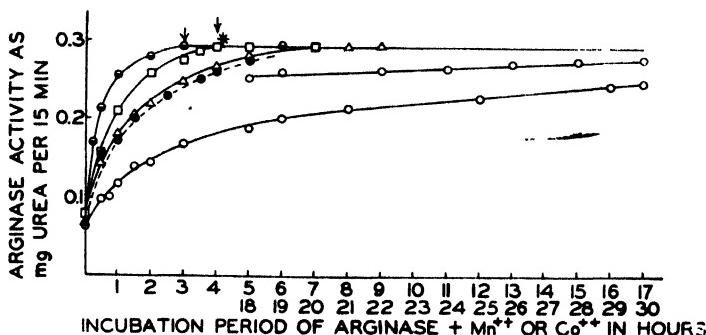


FIG. 3

Effect of Time on Activation of Liver Arginase by Mn<sup>++</sup> or Co<sup>++</sup> at Different Concentrations and Temperatures

- Arginase + 3.3 mg. Mn<sup>++</sup> per ml. at 20°C. Lower row of values of abscissa refer to extension of this curve.
- Arginase + 3.3 mg. Mn<sup>++</sup> per ml. at 40°C.
- Arginase + 1.65 mg. Mn<sup>++</sup> per ml. at 40°C.
- △ Arginase + 0.5 mg. Co<sup>++</sup> per ml. at 40°C.
- Arginase + 0.25 mg. Co<sup>++</sup> per ml. at 40°C.
- ↓ indicates addition of more Mn<sup>++</sup>
- \* indicates addition of more Co<sup>++</sup>

manganese. That the activation of arginase by ions is a time reaction has also been observed by Hunter and Downs (19).

The effect of varying metallic ion concentration for a fixed time interval was determined in another series of experiments. The resulting curves are shown in Fig. 4. Increasing concentrations of Mn<sup>++</sup> or Co<sup>++</sup> are effective in increasing the degree of activity of arginase up to amounts of 1.25 mg. per ml. of enzyme extract. Again it will be noted from Fig. 4 that Co<sup>++</sup> is more potent than Mn<sup>++</sup> at lower concentration. No explanation is available for the difference in the form of the curves for the two ions.

The influence of pH on the activation rate is illustrated by the results recorded in Table IV.

The experiments reported here indicate that the activation of arginase by divalent cations concern the enzyme alone.

The fact that there is a maximum level of activation, which is attained providing enough time is allowed in each case for the reaction to go to completion, is of great interest. After this maximum level of activation is attained the enzyme activity remains constant with further passage of time. The time required to attain the maximum activation is dependent on the kind of ion, its concentration, the pH and the temperature. From the above observations, the action of the metallic cations in the process of arginase activation might be assumed to be purely catalytic were it not for the fact, as will be shown below, that the presence of a metal is necessary for maintenance of the enzymatic activity. Removal of the activating cation, by dialysis, or apparently through formation of complexes with either citrate or borate, causes loss of enzymatic activity. It is also of interest to note

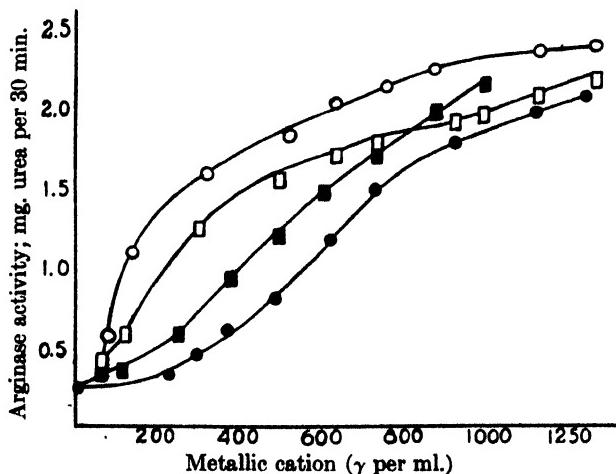
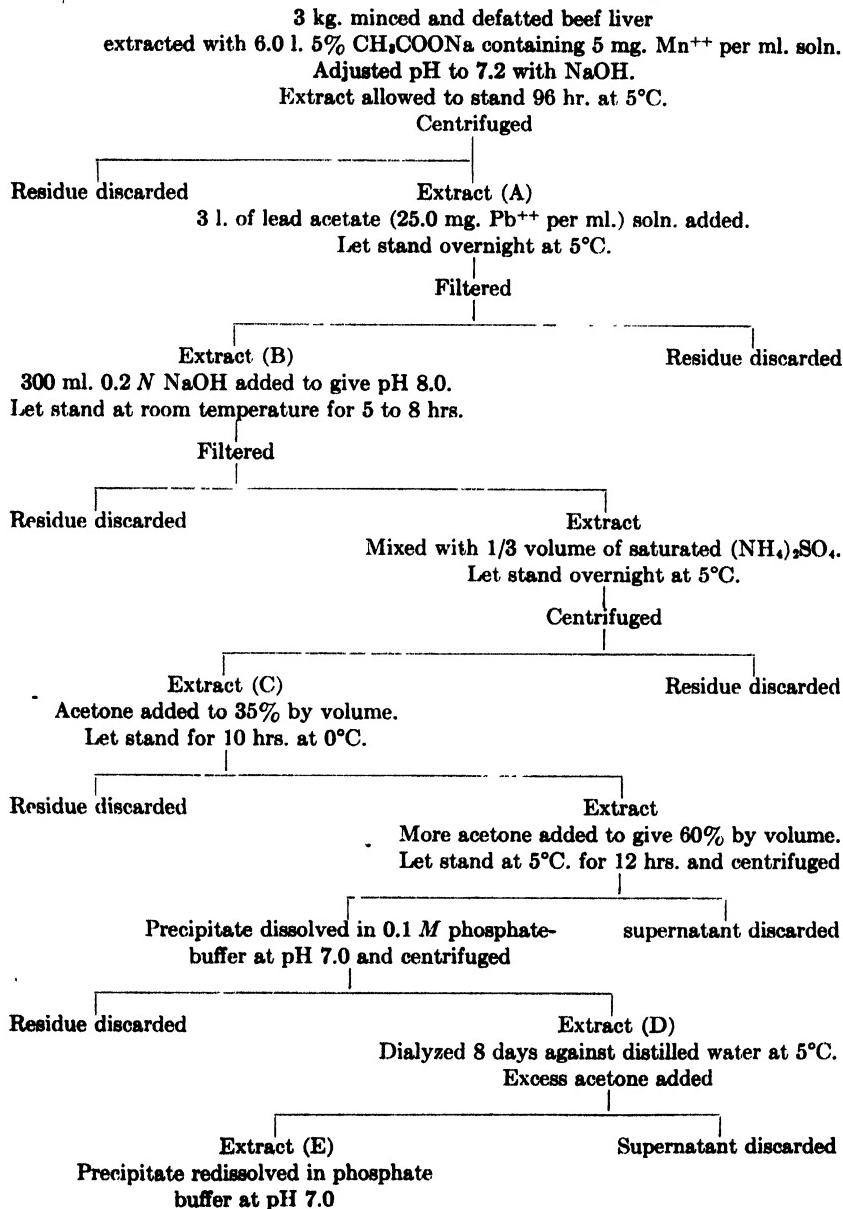


FIG. 4

Effect of the Concentrations of  $Mn^{++}$  and  $Co^{++}$  on the Activation of Liver Arginase at Several Temperatures

- Arginase +  $Co^{++}$  incubated for 60 min. at 20°C.
- Arginase +  $Co^{++}$  incubated for 30 min. at 20°C.
- Arginase +  $Mn^{++}$  incubated for 60 min. at 30°C.
- Arginase +  $Mn^{++}$  incubated for 60 min. at 20°C.

**Scheme for Preparation of Highly Active Liver Arginase**



that the stage of purification of the arginase had no noticeable effect on the activation process.

The explanations offered by other investigators (8, 9) that the arginase activation is due either to the presence of a metallic ion in the structure of the enzyme, or to formation of a co-ordination complex with the metallic ion linking the active group in the arginase molecule with the substrate molecule, do not give a complete picture of the mechanism of the activation.

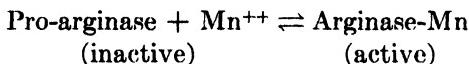
On the basis of the experimental data presented here it is suggested that activation of arginase consists in the transformation of a pro-

TABLE IV  
*Effect of pH on Rate of Arginase Activation by Mn<sup>++</sup>*

Beef arginase, purified by lead acetate and acetone precipitation, was incubated at 40°C. at the indicated pH values with 1.7 mg. Mn<sup>++</sup> per ml. At the stated time intervals, 0.1 ml. aliquots of each pH series were tested for arginase activity by incubating at pH 8.0 and 40°C. for 30 minutes using 0.04 M substrate concentration.

Time of incubation (hours)	<i>Mg. urea per 30 min.</i>		
	pH 6.0	pH 7.6	pH 8.4
0.5	0.153	0.199	0.227
1.0	0.227	0.265	0.332
1.5	0.287	0.325	0.342
2.0	0.335	0.360	0.367
2.5	0.370	0.369	0.370

arginase into active arginase, as represented schematically by the following equation.



The mechanism postulated here is in harmony with all the experimental observations, which are listed below: (1) The partial arginase activity shown by untreated liver extract can be explained by the presence of traces of Mn<sup>++</sup> or Co<sup>++</sup> in the liver. Analysis of rat livers showed the presence of 6.2 γ Mn<sup>++</sup> per g. fresh tissue. The partial activity of the arginase is greatly diminished by dialysis which removes the metal ions. Experiments illustrating this are recorded in Table V. (2) Up to three-fold activation of arginase extracts, can be obtained by the addition of or dialysis against Mn<sup>++</sup> ions (Table V). (3) The reversibility of the activation is shown by the fact that prolonged dialysis reduces the activity of arginase preparations almost to zero.

Activity is also reduced in proportion to their concentration by such anions as citrate and borate (see below). The anions do this, presumably, by forming competing complexes with manganese and cobalt.

The mechanism proposed here does not necessarily conflict with the partial explanation offered by other workers pointing to either the

TABLE V  
*Effect of Introduction and Removal of Activating Ions by Dialysis*

Treatment	Arginase Activity	Total N	Arginase Activity	Total N	Arginase Activity	Total N
	First day		Fifth day		Fifteenth day	
	mg. urea per 30 min.	mg. per ml.	mg. urea per 30 min.	mg. per ml.	mg. urea per 30 min.	mg. per ml.
Extract (1)	0.50	3.55	0.46	3.30	—	—
Dialyzed vs. H <sub>2</sub> O*	0.27	3.30	0.10	—	—	—
Dialyzed vs. Mn <sup>++†</sup>	1.22	3.32	1.24	2.41	1.25	—
Dialyzed vs. H <sub>2</sub> O, then dialyzed vs. Ni <sup>++‡</sup>	0.55	—	0.49	—	—	—
Extract (7)	1.04	3.03	1.24	2.83	1.26	—
Dialyzed vs. H <sub>2</sub> O*	0.38	—	0.17	—	—	—
Dialyzed vs. H <sub>2</sub> O, then dialyzed vs. Mn <sup>++†</sup>	1.20	—	1.19	—	1.19	—
Dialyzed vs. H <sub>2</sub> O, then dialyzed vs. Ni <sup>++</sup>	0.54	1.11	0.49	1.10	0.06	—

\* Glass redistilled water. Extracts (1) and (7) were prepared as in Table II.

† MnSO<sub>4</sub>·4H<sub>2</sub>O solution containing 5 mg. Mn<sup>++</sup> per ml.

‡ NiCl<sub>2</sub>·6H<sub>2</sub>O solution containing 5 mg. Ni<sup>++</sup> per ml.

presence of a metallic ion in the active enzyme molecule or to the action of the metal ion as a link in the formation of a substrate-enzyme intermediate.

#### INHIBITION REACTIONS

*Borate.* The activity of arginase is decreased by the presence of citrate or borate ions. Probably the reason for this is that these acid

TABLE VI

*Effect of Borate Ion on Activity of Liver Arginase*

Arginase purified by lead acetate and acetone precipitation was used for tests. Incubation with  $Mn^{++}$  and activity tests were carried out at 20°C. for 30 minutes.

Solution used for activation with $Mn^{++}$ at pH 7.2	Time of activation with $Mn^{++}$ hrs.	Arginase activity determined in following solutions at pH 7.6	Arginase activity mg. urea per 30 min.
Acetate	0	Phosphate	0.10
Acetate	0	Borate	0.02
Acetate	1	Phosphate	0.22
Acetate	1	Borate	0.03
Acetate	24	Phosphate	0.88
Acetate	24	Borate	0.03
Phosphate	24	Borate	0.03
Phosphate	24	Phosphate	0.18
Borate	24	Borate	0.03

ions form complex compounds with the activating metal ions. The lowering of the concentration of activating metallic ions reduces the enzyme activity. Experiments demonstrating the lowering of the activity of  $Mn^{++}$ -activated arginase in the presence of borate is shown in Table VI. A similar lowering of the enzymatic activity by borate on cobalt-activated arginase is shown in the pH activity curves of Fig. 2.

TABLE VII

*Effect of Heavy Metals on Arginase Activity*

Salts employed were as follows:  $AgNO_3$ ,  $Cu(NO_3)_2 \cdot 2H_2O$ ,  $HgCl_2$ ,  $FeCl_3$ , and  $Pb(NO_3)_2$ . Enzyme activity was determined at 40°C. in pyrophosphate buffer at pH 9.35.

Rat Arginase			Beef Arginase		
Metal ion	Amount	Arginase activity mg. urea per 30 min.	Metal ion	Amount	Arginase activity mg. urea per 30 min.
None	—	0.94	None	—	0.36
$Ag^+$	50	0.69	$Fe^{+++}$	50	0.36
$Ag^+$	250	0.45	$Cu^{++}$	250	0.35
$Ag^+$	600	0.19			
$Cu^{++}$	50	0.94			
$Hg^{++}$	700	0.51			

**Heavy Metal Ions.** Arginase is unusually stable to metal ions. Silver and mercuric ions were the only ones found to be strongly inhibiting. The stability of arginase to lead ions and its use in the purification of the enzyme was discussed earlier. The results of tests on a number of heavy metal ions are recorded in Table VII.

### SUMMARY

1. A method has been developed for preparing liver arginase of high potency. The method is based on extraction of the arginase with sodium acetate solution and on the preferential denaturation and precipitation of non-arginase proteins by lead acetate and acetone. By this method a twenty-fold increase in enzyme activity per mg. of protein nitrogen has been achieved. The isoelectric point of the purified arginase was determined to be in the neighborhood of pH 5.0.

2. Measurements have been carried out of the pH activity curves of the enzyme, with and without ion activators. In an interpretation of these curves, it is suggested that the active enzyme-substrate intermediate is formed by combination of the anionic form of the enzyme and the monovalent cationic form of arginine.

3. The activation of arginase by  $Mn^{++}$ ,  $Co^{++}$  and  $Ni^{++}$  was found to be a reversible reaction governed by time, temperature, pH, the type and the concentration of the activating ion. The maximum activity, once attained, cannot be exceeded by changing the conditions.

4. Arginase is stable in the presence of most heavy metal ions; only  $Ag^+$  and  $Hg^{++}$  were found to be deleterious. Borate and citrate reduce the activity of arginase, probably by competition for the activating metallic ions, with which they presumably form complexes.

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## Liver Arginase

### II. Kinetic Properties

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Received August 22, 1945

#### INTRODUCTION

The kinetic properties of arginase have received but little attention. An investigation of the reaction mechanism was undertaken as a part of the program of the purification and physico-chemical characterization of this enzyme. Certain kinetic properties were observed which appear to be unique in the field of enzymes.

#### *Reaction Order of the Arginine-Aarginase Reaction*

A number of investigators have noted that the hydrolysis of arginine by arginase follows the first order equation for only part of the reaction course. An empirical equation to represent the whole course of the hydrolysis has been developed by Hunter and Morrell (1). One cause of the departure from the first order equation is the inhibiting effect of one of the reaction products, ornithine. Another may be an insufficient content of  $Mn^{++}$  or  $Co^{++}$ . The effect of these ions on the reaction rate is shown by the curves of Fig. 1. The figure shows that with a crude arginase preparation the curve soon departs from that of a first order reaction. On the other hand, in the case of purified arginase, and when  $Mn^{++}$  or  $Co^{++}$  ions are added, the hydrolysis curve is first order throughout almost the whole course of the reaction.

#### *Activity-Substrate Concentration Relations*

The influence of the substrate concentration on the velocity of enzyme catalyzed reactions is widely interpreted in terms of the Michaelis-Menten theory of the

\* This article is derived from a thesis submitted to the Graduate Division of the University of California by Mostafa Safwat Mohamed in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Biochemistry, October, 1943.

formation of an enzyme-substrate intermediate compound (2). The general case for the simplest equilibrium between enzyme and substrate may be represented by the equation

$$K_s = \frac{(E)(S)^n}{(ES_n)} . \quad (1)$$

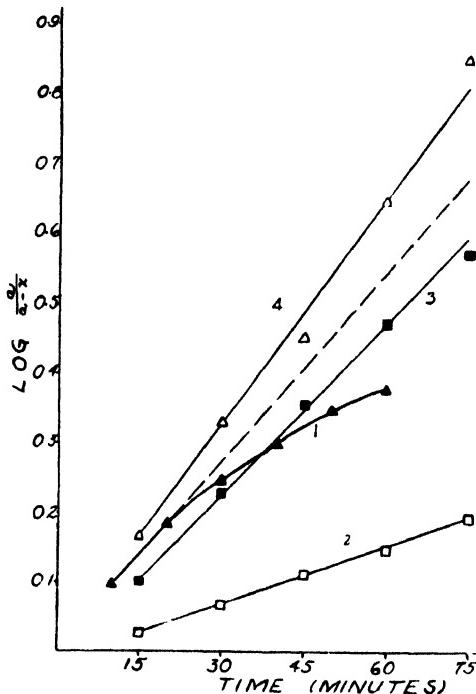


FIG. 1

#### Hydrolysis Reaction of Arginine by Liver Arginase

Curve 1. ▲ Crude beef arginase (sodium acetate extract of liver).

Curve 2. □ Purified arginase, unactivated.

Curve 3. ■ Purified arginase, activated with  $\text{Co}^{++}$  1.33 mg. per ml.

Curve 4. △ Purified arginase, activated with  $\text{Mn}^{++}$  1.33 mg. per ml.

The measurements were carried out at 40°C. and pH 8.5

where  $(E)$  and  $(ES_n)$  are the concentration of free and combined enzyme respectively,  $(S)$  is the concentration of the substrate, and  $n$  is the number of substrate molecules which combine per molecule of enzyme. In all well-authenticated cases,  $n$  has the value of one. Lineweaver and Burk (3) showed that the enzyme-substrate dissociation constant,  $K_s$ , is evaluated most readily by casting the dissociation equation into

the form

$$V_m \frac{(S)}{V} = K_s + (S) \quad (2)$$

In the above equation  $V_m$  represents the maximum velocity,  $V$  the velocity for a short time interval and  $(S)$  the substrate concentration.

It is apparent that if the equation is obeyed, a plot of  $\frac{(S)}{V}$  against  $(S)$  will yield a straight line, in which the inverse of the slope, namely

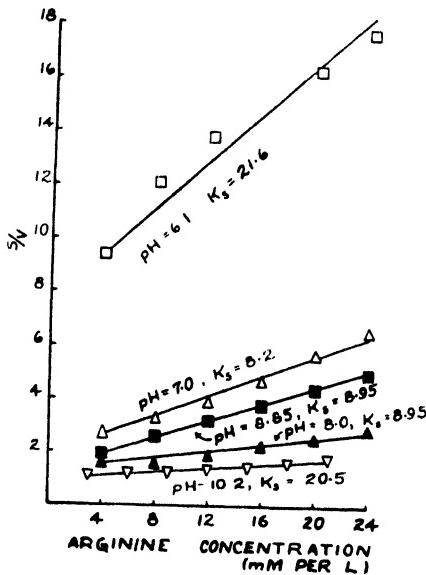


FIG. 2  
Substrate Concentration-Activity Curves of Beef Liver Arginase.  
Influence of pH on the Michaelis-Menten Constant

Purified arginase prepared by the lead acetate and acetone precipitation method employed. Enzyme activity was determined at 40°C. and the reaction time was 15 min.; pH and  $K_s$  values are given in the figure.

$d(S)/d \frac{(S)}{V}$ , represents the maximum velocity,  $V_m$ , and the intercept on the  $(S)$  axis, i.e., the point where  $\frac{(S)}{V} = 0$ , is the negative value of the constant  $K_s$ .

Plots of such curves at a number of pH values for beef arginase and for cobalt-activated rabbit arginase are given in Figs. 2 and 3. The

plots show that the data fit the straight line relationship very well indeed. Examination of the plots show that the values of  $K_s$  are not absolute constants but, instead, are dependent upon the pH. It is also to be noted that, with changing pH, the values of  $K_s$  pass through a minimum.

A consideration of the nature of the arginine-arginase reaction, and

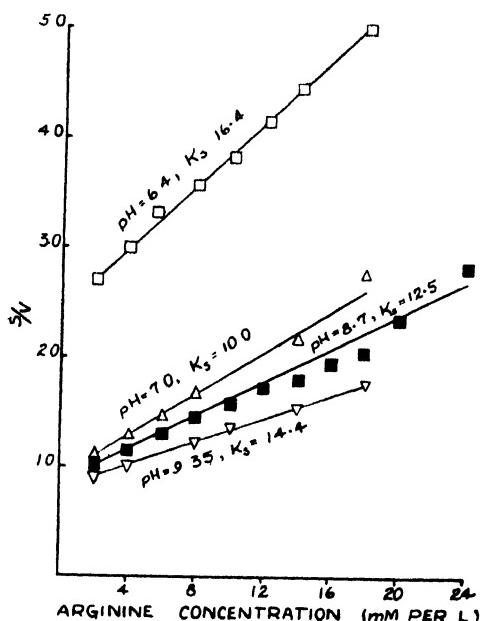


FIG. 3

Substrate Concentration-Activity Curves of Cobalt-Activated Rabbit Liver Arginase. Influence of pH on the Michaelis-Menten Constant

Arginase extracted with 5% sodium acetate solution containing 5 mg.  $\text{Cu}^{++}$  per ml. and purified by acetone fractionation; enzyme activity determined at 40°C.; reaction time 30 min.

the influence of pH upon it offers a theoretical explanation for the change of  $K_s$  with pH. This is brought out by the following derivation. The discussion of the nature of the activity-pH curve of arginase developed that the monovalent cation of arginine and the anionic form of arginase are the probable actual reactive components. Representing these by  $A^+$  and  $E^-$ , one may write for the enzyme-substrate

dissociation equation,

$$\frac{(E^-)(A^+)}{(EA)} = K_s \quad (3)^1$$

For the acid dissociation of the arginine one may write the equation



This leads to the mass law equation

$$\frac{(C_H)(A^\pm)}{(A^+)} = K_1 = 9.12 \times 10^{-10} \quad (5)$$

Let

$$(A) = (A^\pm) + (A^+) \quad (6)$$

Then

$$\frac{C_H[(A) - (A^+)]}{(A^+)} = K_1 \quad (7)$$

And

$$(A^+) = (A) \left( \frac{C_H}{K_1 + C_H} \right) \quad (8)$$

Applying the same treatment to the dissociation of the enzyme there is obtained



And

$$\frac{(E^-) \cdot C_H}{(E^\pm)} = K_2 \quad (10)$$

Then

$$(E^\pm) = \frac{(E^-) \cdot C_H}{K_2} \quad (11)$$

Let

$$e = E^- + E^\pm + EA \quad (12)$$

Then

$$e - (EA) = (E^-) \left( 1 + \frac{C_H}{K_2} \right) \quad (13)$$

Combining equations (3), (8) and (13) there is obtained

$$\frac{[e - (EA)] \cdot (A) \left( \frac{C_H}{K_1 + C_H} \right)}{\left( 1 + \frac{C_H}{K_2} \right) (EA)} = K_s \quad (14)$$

Hence

$$(EA) = \frac{e(A) \left( \frac{C_H}{K_1 + C_H} \right)}{\left( \frac{C_H}{K_1 + C_H} \right) + K_s \left( 1 + \frac{C_H}{K_2} \right)} \quad (15)$$

Now using the relationships that

$$V = K(EA) \quad \text{and} \quad K_e = V_m$$

<sup>1</sup> An analogous treatment of the kinetic behavior of the enzyme histidase has been employed by Walker and Schmidt (6).

There is obtained

$$V = \frac{V_m(A) \left( \frac{C_H}{K_1 + C_H} \right)}{A \left( \frac{C_H}{K_1 + C_H} \right) + K_s \left( 1 + \frac{C_H}{K_2} \right)} \quad (16)$$

Rearranging gives

$$\frac{V_m(A)}{V} = (A) + K_s \left( \frac{K_1}{C_H} + 1 \right) \left( \frac{C_H}{K_2} + 1 \right) \quad (17)^*$$

As in equation (2), it follows that if equation (17) is obeyed, a plot of  $\frac{(A)}{V}$  against  $(A)$  yields a straight line. Extrapolation to the value of  $\frac{(A)}{S} = 0$ , leads to the result that  $-A = K_s$  (exp.), and

$$K_s(\text{exp.}) = K_s(\text{true}) \left( \frac{K_1}{C_H} + 1 \right) \left( \frac{C_H}{K_2} + 1 \right) \quad (18)^*$$

From equation (18), it follows that the experimentally observed  $K_s$  will not be a constant, but instead will be a function of the pH. The true enzyme substrate dissociation constant,

$$K_s(\text{true}) = \frac{K_s(\text{exp.})}{\left( \frac{K_1}{C_H} + 1 \right) \left( \frac{C_H}{K_2} + 1 \right)} \quad (19)$$

The value of  $K_1$  representing the dissociation of arginine, can be obtained from compilations of the dissociation constants of arginine and has the value of  $pK_1 = 9.04$  or  $K_1 = 9.12 \times 10^{-10}$  (4). From the consideration of the pH activity curve of arginase it was deduced that  $K_2$  has the approximate value of  $10^{-7}$  (5).

If these figures are used the following magnitude is obtained for the

\* It will be noted that equation (17) is essentially similar to equation (2), differing only by the terms for the superimposed effect of the hydrogen ion concentration on the dissociation of enzyme and substrate.

<sup>†</sup> That  $K_s(\text{exp.})$  passes through a minimum at  $\sqrt{K_1 K_2}$  is readily shown by means of the differential calculus in the following manner:

$$\frac{d(K_s(\text{exp.}))}{d(C_H)} = K_s(\text{true}) \left[ \left( \frac{K_1}{C_H} + 1 \right) \frac{1}{K_2} - \left( \frac{C_H}{K_2} + 1 \right) \frac{K_1}{C_H^2} \right] = 0$$

Then

$$K_s(\text{true}) \left( \frac{1}{K_2} - \frac{K_1}{C_H^2} \right) = 0$$

and

$$C_H = \sqrt{K_1 K_2}$$

isoelectric point of the arginine-arginase intermediate, which also represents the point of minimum for  $K_s$  (exp.); namely,

$$pI = \frac{1}{2}(7.0 + 9.04) = 8.0 \text{ (approximately).}$$

Plot of the experimental  $K_s$  values against pH yields a U shaped curve in which it will be noted that the minimum falls at about pH 8.0 (Fig. 4). This has also been noted by Van Slyke (7). A value

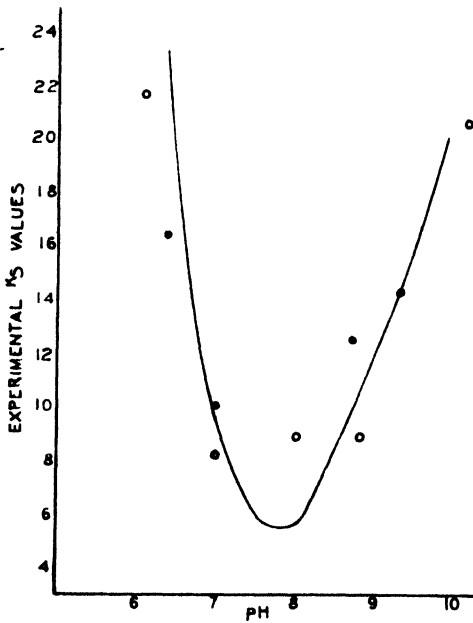


FIG. 4

Variation of the Michaelis-Menten Constant of Liver Arginase with pH  
 ○ beef arginase.      ● rabbit arginase.

of  $K_s$  (true) = 4.5 mM (in terms of arginine concentration) was calculated from equation (19), using the constants given above.

#### TEMPERATURE EFFECTS

*Optimum Temperature.* Curves of the change of arginase activity with temperature are shown in Fig. 5. The curves show that the temperature of optimum activity of the enzyme is at about 50°C. In this respect arginase behaves like most enzymes of animal origin.

The temperature-activity curve obtained in buffered citrate solution is interesting as being another example of the inhibiting action of this ion. Although it was much closer to the pH of optimum activity, the reaction rate in the citrate solution was a good deal lower at all temperatures than it was in the phosphate buffer of pH 7.5.

*Kinetics of Heat Inactivation.* Study of the rate of thermal inactivation is of considerable value in characterizing an enzyme. The velocity constants, half lives and critical thermal increment derived from such

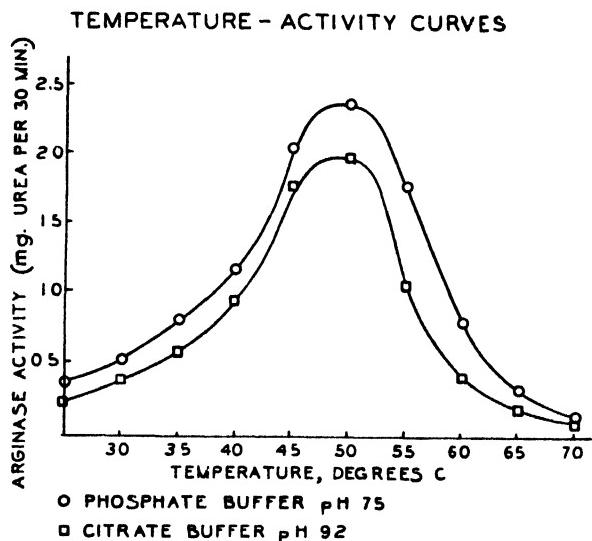


FIG. 5  
Temperature-Activity Curves of Rat Liver Arginase.  
Preparation obtained by acetone fractionation

rate studies are a measure of the degree of thermal stability of an enzyme.

The thermal inactivation of arginase, as has been observed for most enzymes, obeys the equation of a first order reaction. This equation is commonly written as

$$2.3 \log A_0/A = kt \quad (20)$$

where  $A_0$  is the initial activity of the unheated enzyme solution,  $A$  the residual activity after heating for time  $t$ , and  $k$  is the velocity constant. The straight lines obtained by plotting the logarithm of the residual

activity against time for the inactivation of arginase at a number of different temperatures are shown in Fig. 6.

The critical thermal energy for the inactivation of the enzyme and the heat of activation for the arginine-arginase reaction were calculated

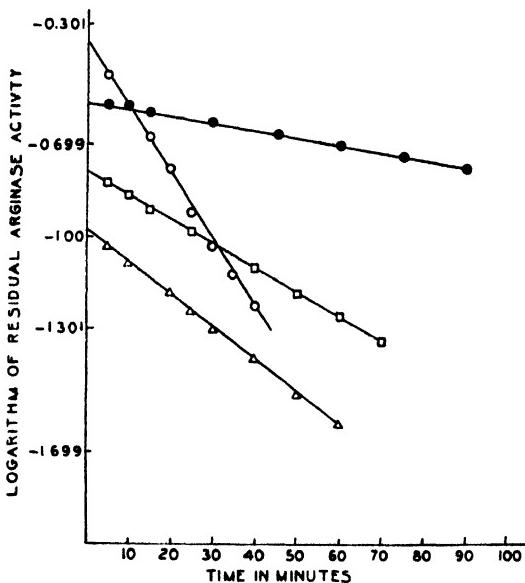


FIG. 6

Rate of Heat Inactivation of Arginase. (Rat liver arginase extracts employed) Phosphate buffer of pH 7.4 at 50°, 55° and 60°C., and buffered citrate solution of pH 9.35 at 55°C. used. First order constants  $\times 100$  from the slopes are 0.53 at 50°C., 1.9 at 55°C., and 2.4 at 60°C. in phosphate buffer, and 5.0 at 55°C. in citrate solution.

from the data plotted in Figs. 5 and 6 with the aid of the Arrhenius equation

$$\frac{d \ln k}{dT} = \frac{E}{RT^2} \quad (21)$$

This equation relates the velocity constant  $k$ , the absolute temperature  $T$ , and the heat of activation of the reaction or the inactivation of the enzyme. Since  $\ln k$  is also proportional to the logarithm of the reaction velocity, the energy values can be evaluated from a plot of the log of the enzyme activity against  $1/T$ . The slope of the straight line has

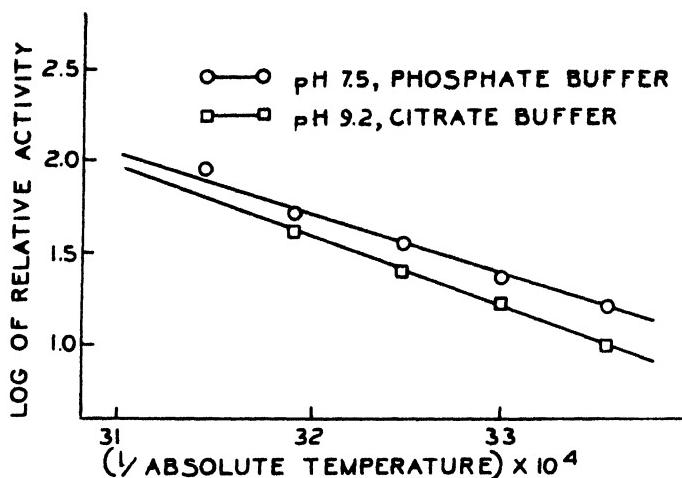


FIG. 7

Relation Between Velocity of Arginine-Arginase Reaction and Absolute Temperature. Plotted from Data of Fig. 5

Activation energy from slopes = - 13,850 calories per mole in phosphate buffer (pH 7.5) and - 16,000 calories per mole in buffered citrate solution (pH 9.2).

the value  $E/4.56$ . Such plots for the range of temperature of increasing reaction rate are given in Fig. 7, and plots for the range of temperature over which the enzyme is inactivated are given in Fig. 8.

#### SUMMARY

1. The hydrolysis of arginine by crude arginase does not follow a first order reaction. With purified enzyme and in the presence of manganous and cobaltous ions as activators, the reaction closely obeys the first order reaction equation.
2. Analysis of the change of reaction with substrate concentration indicated that the enzyme-substrate intermediate apparently consists of one molecule of arginase combined with one molecule of arginine at all the pH values tested. The enzyme-substrate dissociation constant (Michaelis-Menten constant) varied with pH in a manner which yielded a U shaped curve, the minimum value being at about pH 8.0. This curve has been interpreted in terms of the dissociation with respect to pH of both the substrate and the enzyme. Evaluation of

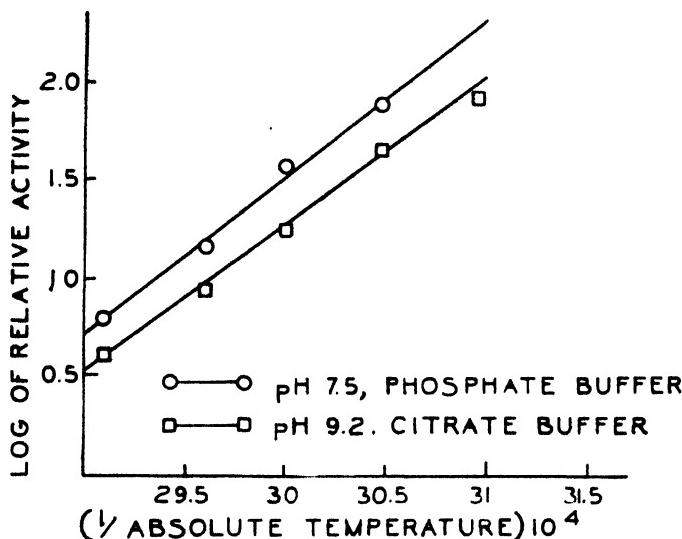


FIG. 8

Relation Between the Rate of Inactivation of Arginase and the Absolute Temperature. Plotted from Data of Fig. 5

Energy of inactivation from slopes = 35,600 calories per mole in phosphate buffer (pH 7.5) and 34,250 calories per mole in buffered citrate solution (pH 9.2).

the true enzyme-substrate dissociation constant yielded the value of  $4.5 \times 10^{-8}$ .

3. The optimum temperature for the activity of arginase was observed to be between 45 and 50°. The thermal inactivation of arginase follows a first order reaction. The critical thermal increment of the enzyme inactivation and the heat of reaction have been calculated from the kinetic data.

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## Penicillin

### VII. Penicillinase<sup>1</sup>

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Received July 5, 1945

#### INTRODUCTION

At the time this work was started (July 1943) the only previous references to enzymatic inactivation of penicillin were those of Abraham and Chain (1), who had shown that various bacterial extracts and filtrates were capable of destroying penicillin, and Hobby *et al.* (2), who confirmed the former's observation with respect to *Escherichia coli*. Since that date, many new observations have been made concerning penicillin-destroying enzymes. Lawrence (3) stated that takadiastase and clarase were effective in the destruction of penicillin. The same investigator (4) later reported that clarase inactivation of penicillin was due to certain filterable substances of bacterial origin. That these penicillin-destroying organisms were probably chance contaminants not regularly present in the production of clarase was shown by Stanley (5), who found only one of five lots of clarase to possess penicillinase activity. Penicillinases from various bacterial sources have received the most attention, and in certain cases have been extracted from the cells with various solvents by Harper (6), Kirby (7), Ungar (8), and McQuarrie *et al.* (9). It has been reported by Bondi and Dietz (10) that a large variety of bacteria produce penicillinase.

Woodruff and Foster (11 and 12) found that penicillinase activity was not limited to bacteria, inasmuch as certain yeasts, actinomycetes and other fungi formed it in varying degrees. Himes and White (13) tried a large number of substances, including growth factors, amino acids, carbohydrates, phospholipids, purines, tissue extracts and certain bacterial filtrates, as penicillin inactivators; but, with the exception of the latter, all proved ineffective.

The primary purpose of these investigations was to find an agent capable of destroying penicillin prior to testing for its sterility. Part I

<sup>1</sup> The expense of the work described in this paper was met in part from Contract OEMcmr-100 with the Office of Scientific Research and Development, recommended by the Committee on Medical Research.

<sup>2</sup> One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

of this paper deals briefly with investigations on various liquid and dehydrated enzyme preparations, and part II treats of experiments with certain bacterial extracts and the preparation of a powerful exocellular penicillinase.

### *I. Effect of Various Enzyme Preparations on Penicillin*

The general method for testing enzyme preparations for their penicillin-destroying ability was that described below for takadiastase. Half of a 1% solution of takadiastase in a weak phosphate buffer at pH 7.0 was Seitz-filtered, and the remainder was left untreated. Buffer solutions at pH 4.0, 5.0, 6.0, 7.0 and 8.0 were saturated with chloroform and toluene to prevent the growth of microorganisms. The tests were set up so that each tube contained 4.2 ml. of buffer, 0.5 ml. of 1.0% takadiastase and 0.3 ml. of penicillin (100 Oxford units). The Seitz-filtered and unfiltered takadiastase-buffer-penicillin tubes were incubated for 4 hours at 37°C. along with penicillin-buffer controls before dilution for assay by the cylinder-plate method of Schmidt and Moyer (14). Destruction of penicillin in the controls at pH 4.0 and 8.0 was attributed to the increasing instability of the drug above and below pH 6.0, as previously shown by Benedict *et al.* (15). No appreciable loss of penicillin occurred at pH 5.0 to 7.0 with Seitz-filtered or unfiltered takadiastase.

A number of enzyme preparations tested in the manner described above are listed in Table I. Most of these enzymes, plus some additional ones, were tested for penicillinase activity with comparable results by Lawrence (16). Slight penicillin destruction occurred with a pumpkin protease concentrate prepared by the method of Willstätter *et al.* (17) and considerable inactivation by a high diastatic malt sirup. A Seitz-filtered preparation of this sirup had been substituted for corn steep liquor in a medium intended by Moyer and Schmidt for penicillin production. Good mold growth occurred, but no penicillin accumulated. This was in contrast to behavior of the same malt sirup which had been heat sterilized and which permitted good growth and penicillin accumulation. The authors found that 1.0 ml. of 10% sirup destroyed 250 units of penicillin in 4 hours at 37°C. In purification and concentration experiments too numerous and detailed to be mentioned here, a preparation was finally obtained which was 40 times as potent as the original. The difficulty of concentrating the original

TABLE I  
*Effect of Various Enzyme Preparations on Partially Purified Penicillin*

Name	pH	Final concentration of enzyme	Temp.	Time of incubation (Hours)	Penicillin destruction
		Per cent	°C.		
Takadiastase, Seitz-filtered or untreated	4.0, 5.0, 6.0,	0.1	37	4	None
	7.0 and 8.0				
Papain*	4.0, 5.0 and	0.1	37	2.5	None
	6.0				
Urease	7.0	0.1	37	3.0	None
	Fresh hog duodenal mucosa (peptidases)				
Polidase, a diastatic mixture	7.0 and 8.0	—	37	4.0	None
	6.0 and 7.0				
Emulsin ( $\beta$ -glucosidases)	5.0	0.2	25	4.0	None
	6.0				
Concentrated pumpkin protease	6.3, 7.0 and	—	30	3.0	None
	7.2				
Ficin†	6.0 and 7.0	0.1–1.0	30	4.0	None
	8.0				
Trypsin	Pure carbonic anhydrase‡ plus 0.05% peptone	0.5	40	4.0	None
	6.0				
Crude $\alpha$ -amylase	5.0	—	40	3.0	None
	5.0				
Crude $\beta$ -amylase	5.0	—	40	3.0	None
	5.0, 6.0 and				
High diastatic malt syrup <sup>1</sup>	7.0	—	37	4.0	100%

\* Activated with cysteine-HCl for 1.5 hours at 37°C.

† Received through the courtesy of Dr. M. J. Johnson, University of Wisconsin.

‡ Received through the courtesy of Dr. D. A. Scott, Connaught Laboratories, Toronto.

<sup>1</sup> Blue Ribbon high diastatic malt syrup made by the Pabst Brewing Corp., Peoria, Ill.

material made this preparation impractical for sterility testing of penicillin.

## *II. Preparation of a Powerful Exocellular Penicillinase*

Sixty-five bacterial cultures were selected from the culture collection of this Laboratory and were tested for penicillinase activity. Included in this group were species of *Serratia*, *Escherichia*, *Aerobacter*, *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Proteus*, and *Bacillus*. A few strains

were able to initiate growth in the presence of 200 units of crude penicillin per ml. When these organisms were grown in nutrient broth, with or without penicillin, and the bacterial cells removed by centrifugation or Seitz filtration, it was impossible to demonstrate the presence of a penicillin-destroying agent in the broth filtrate. In the case of the organisms grown in the presence of penicillin, it appeared that the inactivating agent remained in the cells as an endoenzyme.

At this stage of the problem the authors isolated a chance contaminant which was encountered in penicillin-production flasks. The medium in the flasks, used for the submerged production of penicillin, consisted of corn steep liquor, lactose and mineral salts (18). The two lightly contaminated flasks, although normal in appearance and odor, were found to contain an exocellular penicillinase so potent that one part in 5000 (0.0002 ml. per ml.) would destroy 50–60 units of penicillin per ml. at pH 6.0 within 3 hours at 25°C.

Morphological and cultural studies made on the contaminants isolated from the flasks showed them to be closely related to, or identical with, *Bacillus cereus*. The cultures were added to our bacterial collection as NRRL B-568 and NRRL B-569.

Our first attempt to prepare a penicillinase with the aid of B-569 failed because we did not anticipate the delicate balance which must exist between the developing mold mycelium and the bacterial cells. If too many bacteria were added after inoculation with the mold spores, little or no mycelial growth took place. After good development of the mold mycelium with its attendant penicillin formation, the addition of large numbers of B-569 had no destructive effect.

Successful enzyme production was finally obtained by the following procedure: A heavy 24-hour broth culture of B-569 was diluted in series with sterile distilled H<sub>2</sub>O to 1 × 10<sup>-8</sup> and cell counts were made on agar plates. One ml. from each dilution was added to a shaker flask containing 125 ml. of the penicillin medium previously mentioned, which had been inoculated with mold spores of *P. notatum* NRRL 832 or *P. chrysogenum* NRRL 1951.B25. It is preferable to allow the mold one day's growth on a shaking machine at 24°C. before adding the bacterial cells. Table II shows the results of a typical experiment.

The penicillinase content of the culture liquor was determined by serial dilution. The standard test included enzyme dilutions at 1:1000, 1:3000 and 1:5000 in 1.5% phosphate buffer at pH 7.0. The final concentration of penicillin was 100 units per ml. The tubes were incubated for 4 hours at 30°C. and then diluted for assay. Under proper conditions of preparation it was not difficult to obtain a crude enzyme of which 0.0002 ml. would completely destroy 100 units of penicillin in 4 hours at pH 7.0 and 30°C.

TABLE II

*Effect of Varying Amounts of Bacterial Inoculum on Penicillinase Production*

Flask No.	Approximate number of bacteria added to each shaker flask	Enzyme production after 5-7 days
1	80,000,000	None
2	8,000,000	None
3	800,000	Poor
4	80,000	Fair
5	8,000	Good
6	800	Excellent
7	80	Excellent
8	8	None

Maximum enzyme production occurred at 5-7 days at 24°C. The crude culture liquor was filtered through canvas on a Büchner funnel to remove the mycelium. After centrifugation to remove bacterial cells, 25 ml. lots were lyophilized. The dried preparation resembled Difco Peptone in appearance and was very hygroscopic. Filtration of the culture liquor through Seitz filters caused heavy losses in potency. The lyophilized material appeared to be fairly stable. Partial purification was effected by dialyzing the crude culture liquor in Visking sausage casings against slowly running distilled H<sub>2</sub>O at 0.5°C. for 2-3 days. When retested after lyophilizing, the resulting material had lost very little activity. One mg. destroyed 100 times its own weight of pure penicillin G (1667 Oxford units per mg.) in 3 hours at pH 7.0 and 30°C. An arbitrary penicillinase unit was established as the minimum amount of enzyme which will destroy 50% of 100 Oxford units of crystalline penicillin in 1.0 ml. of pH 7.0 phosphate buffer in 3 hours at 30°C. One unit was found to be 0.0003 mg. of the preparation described above.

### *III. Nature of Action of Penicillinase*

It is of some interest to speculate on the nature of the action of penicillinase. Experiments were accordingly carried out in an effort to ascertain the character of the group in the penicillin molecule which was being attacked. Since this work was completed, Foster (19) has shown that a cell-free bacterial penicillinase preparation acted on penicillin in the presence of bicarbonate with the concomitant evolution of CO<sub>2</sub>. The presumption is that a new acidic group is being liberated by the action of the enzyme on penicillin.

To determine the nature of the acid group formed by the action of the enzyme on pure penicillin, samples of sodium penicillin G were treated with enzyme, and electrometric titrations were carried out on the inactivated solutions. Interpretation of the titration curves indicates that the pK of the acid group formed during the enzyme reaction is 4.70, and that of the acid group originally present is 2.16. This latter value is in reasonable agreement with the pK of penicillin.

To demonstrate that the acidity of the reaction mixture was not due to the enzyme preparation itself a blank determination was made on a solution containing only water and the enzyme preparation. Titration of this solution required only a negligible quantity of alkali.

In general, enzyme properties are known to change markedly as the impurities are removed. Detailed studies on the chemical properties of the penicillinase were not made beyond that previously mentioned. However, the following additional facts were noted concerning the crude dialyzed preparations: (1) More than 50% of the activity was destroyed in 30 minutes at 60°C. in pH 7.0 buffer. (2) The optimum pH was between 6.5 and 7.0 at 25°C. Tests run at pH 5.0 indicated that the enzyme was only half as active, and at pH 8.0, only 5% as active as at pH 7.0. (3) The destruction of crystalline penicillin G by the crude enzyme was very rapid. Three mg. were found by Stodola, MacMillan and Scott (20) to destroy more than half of 60,000 Oxford units in 8 minutes at pH 6.6 and 25°C. The crude enzyme was not poisoned by sodium azide or iodoacetic acid when penicillin in crude culture liquor was used as the substrate. These two chemicals were found to inhibit the exocellular penicillinase described by Woodruff and Foster (11). In our case the activity of these compounds may have been inhibited by the impurities of both substrate and enzyme preparations.

#### DISCUSSION

Although numerous substances have been tried as penicillin inactivators, those of enzymic nature which have proved effective appear, according to Woodruff and Foster (11), to be limited to certain bacteria and to some yeasts, actinomycetes and other fungi. When this work was initiated, little was known of the chemical nature of the product for which an effective destruction agent was sought, and a wide variety of enzyme preparations tested were inactive against penicillin. The attempted destruction of penicillin by urease was made before

Turner, Heath and Magasanik (21) reported this enzyme to be inhibited by penicillin and suggested that this fact might afford a basis for assay of the latter. Scudi and Jelinek (22) subsequently showed that crude penicillin inactivated urease, whereas crystalline penicillin did not.

The weak activity shown by the 10% malt sirup may have been due to aging because it had been received and kept at 40°F. for 22 months before the present tests were made. However, this could not be definitely established, as the company had discontinued the manufacture of this particular type of sirup before this work began.

Although the extraction of penicillinase from bacterial cells with acetone and other solvents has been used by other workers to fair advantage, many of the resulting preparations are weak. One method reported in the literature gave yields as low as 100 units of penicillin destroyed per mg. of dried enzyme under optimum conditions. The method of penicillinase production with *Bacillus cereus* NRRL B-569 is simple and provides a product 10-20 times as potent as any yet described. It is easily standardized according to Liebmann *et al.* (25), who have produced the penicillinase with B-569 by a slight modification of our procedure.

### SUMMARY

Papain, lysozyme, urease, peptidases from porcine duodenal mucosa, polidase, emulsin, ficin, trypsin, pure carbonic anhydrase, crude  $\alpha$ - and  $\beta$ -amylase and one lot of takadiastase were inactive as destroyers of penicillin. Concentrated pumpkin protease and high diastatic malt sirup showed some destructive activity.

A method is described for the production of a very potent exocellular penicillinase by using *Bacillus cereus* NRRL B-569. One mg. of this product will completely destroy 165,000 units of crystalline penicillin in 3 hours at pH 7.0 and 30°C.

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# The Activation of Papain and Related Plant Enzymes with Sodium Thiosulfate

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Received July 17, 1945

## INTRODUCTION

A considerable number of substances have been studied as possible activators for papain. These studies led to the development of theories on the mode of action of these activators. As we found sodium thiosulfate to be a potent activator for papain and especially suitable for the study of the activation reaction, we shall present here some of our results. Moreover, thiosulfate may have some practical interest in connection with the industrial and medical use of papain.<sup>1</sup>

## MATERIALS AND METHODS

The ferments used were mostly crude preparations. Papain and chymopapain were used both in the crude form and also after purifying according to the procedure of Balls and Lineweaver (2) or Jansen and Balls (3), respectively. However, our preparations were not crystallized. Ficin was obtained from samples of ficus sap by precipitation with acetone, and purified by redissolving and reprecipitating. In the same way, tabernamontanain (1) and the ferment from *calotropis gigantea* (4) were obtained. The latter will be referred to as "calatropain." Bromelin was prepared from pineapple juice according to the method of Balls, Thompson and Kies (5). The ferments were stored in well-stoppered glass bottles. For the experiments, freshly prepared 7.5% solutions were used. If necessary, these were diluted with reboiled distilled water.

The ferment activity was determined by the milk clotting method of Balls and Hoover (6) or the formal titration method. In the latter case, gelatin or peptone were used as substrates. Not all the experiments gave reproducible results. Only

<sup>1</sup> After the experimental work referred to in this paper had been finished, Dr. R. T. Major was so kind as to inform us that thiosulfates had already been described as activators for papain by Nies-Harteneck in German Patent No. 532,398. A preliminary note of our findings on the activation of papain by sodium thiosulfate has been included in a previous paper (1).

those results duplicated with at least two different enzyme preparations are mentioned in the following.

## RESULTS

The thiosulfate activation of the milk clotting action of 6 ferments of the papain group has been checked. The results are presented in Table I. These preparations were all partly inactivated by storage or treatment with  $H_2O_2$ . In every case, activation could be caused by

TABLE I  
*Activation of the Milk Clotting Activity of Different Ferments*

Ferment	Activator	Clotting Time minutes
Papain	—	1.25
Papain	thiosulfate	0.57
Chymopapain	—	12.00
Chymopapain	thiosulfate	0.83
Ficin	—	2.75
Ficin	thiosulfate	0.42
Ficin	cysteine	0.46
Bromelin	—	15.00
Bromelin	thiosulfate	6.30
Tabernamontanain	—	2.33
Tabernamontanain	thiosulfate	0.42
Calatropain	—	60.
Calatropain	thiosulfate	1.75
Calatropain	cysteine	1.68

0.1 cc. of a 7.5% ferment solution was mixed with 0.1 cc. of a 1/200 M activator solution and kept for 5 minutes at room temperature. Then 5 cc. of milk, prepared according to Balls and Hoover (6), at 40°C. were added to the mixture and the liquid kept at this temperature in a water bath until clotting occurred.

adding thiosulfate. The varying degrees of activation may possibly be a function of the ratio between reversible and irreversible inactivated enzyme present in each preparation and need not be considered as a characteristic of the different ferments.

The chymopapain, bromelin and calatropain preparations gave negative nitroprusside tests which remained negative after activation with thiosulfate.

Willstätter and Grassmann considered the activation of papain with cysteine to be a time-reaction (7). Scott and Sandstrom recently questioned Willstätter's finding (8). We present evidence in Table II

that activation of the milk clotting action of papain with cysteine, KCN,  $\text{Na}_2\text{S}$  and thiosulfate are time-reactions. Both the ferment solution and the activator solution were cooled to 5°C. They were then mixed, and milk at 40°C. added and the clotting time determined in the usual manner. In experiments 5-8 the ferment-activator solution was kept at 40°C. for 3 minutes before the milk was added. No full activation took place without incubation. The effect of incubation could be observed to a lesser degree with uncooled ferment-activator solutions. If a substantial excess over the minimum amount of activator necessary to produce full activation is employed, however, the effect is scarcely detectable.

TABLE II  
*Determination of the Influence of Incubation on the Activation of Ficin*

No.	Activator	Clotting Time minutes	Determination
1	—	4.25	
2	cysteine	3.85	
3	KCN	3.33	
4	$\text{Na}_2\text{S}$	3.00	
5	thiosulfate	2.70	
6	cysteine	1.00	
7	KCN	0.92	
8	$\text{Na}_2\text{S}$	1.18	incubated 3 minutes
9	thiosulfate	0.83	

The cooled activator solution was added to the cooled ferment solution and the clotting time determined immediately, or after 3 minutes incubation at 40°C.

The quantitative relation between ferment and activating sodium thiosulfate was also studied. In Fig. 1 the result of such an experiment is presented. Curve 1 represents the relationship between the reciprocal clotting time and the amount of enzyme. It is virtually identical with the curves described by Balls and Hoover for papain (6). Curve 2 was obtained by plotting the reciprocal clotting time against the amount of thiosulfate used to activate 7.5 mg. of ficin. This curve shows that the activation is a reaction occurring in stoichiometric fashion. If the molecular weight of ficin is considered to be similar to that of papain—near 30,000—curve 2 indicates that equimolar amounts of thiosulfate activate equimolar amounts of ficin. The deviation of curve 2 from curve 1 is probably caused by the action of the residual inactive ficin. Balls and Hoover have shown that amounts of ferment

not sufficient to produce coagulation of a given amount of milk, will nevertheless effect later clotting of this same milk with a smaller amount of active enzyme than usual. This same effect probably causes curve 2 to deviate from curve 1.

There is a possibility that one molecule of ficin is activated by more than one molecule of thiosulfate, which could be concealed by impurities in the ferment preparation or by a higher molecular weight of ficin. But analogous results were obtained with a preparation of papain, which excludes this possibility.

The method of stoichiometric activation can be used to determine

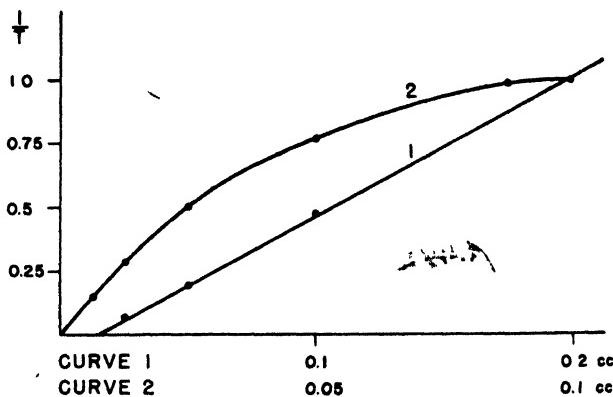


FIG. 1

Relationship between clotting time (reciprocal) and amount of activated ficin.  
Curve 1. 3.25% thiosulfate activated ficin solution. Curve 2. 1/400 M  
thiosulfate solution added to 0.1 cc. of 7.5% ficin solution.

the degree of purity in crude ferment preparations. In pure preparations, it can serve to detect the degree of reversible inactivation. In pure and reversible inactivated preparations, it may serve for crude determination of molecular weight in a manner similar to the method of quantitative inactivation with iodoacetic acid used by Balls and Lineweaver (2).

The speed of the reduction of S-S linkages to thiol groups depends upon the pH of the solution (9, 12). The reduction of S-S proteins is not believed to occur in an acid solution (10). Therefore, we checked whether activation would occur in a dilute acid. We mixed equal

volumes of ferment solution and  $N/10$  HCl. The activator was then added and milk clotting time immediately determined with and without previous neutralization. The speed of the activation was not diminished to any detectable degree in the acid solution. The activities of the

TABLE III  
*Activation of Ficin in Acid Medium*

Activator	$N/10$ HCl	$N/10$ NaOH	Clotting Time minutes
$\text{Na}_2\text{S}$	—	—	1.00
	0.1 cc.	—	0.58
	0.1 cc.	0.1 cc.	0.75
$\text{Na}_2\text{S}_2\text{O}_3$	—	—	0.92
	0.1 cc.	—	0.58
	0.1 cc.	0.1 cc.	0.88
—	0.1 cc.	—	4.10
—	—	—	4.30

The acid was added to the ferment solution before the activator solution. Neutralization was carried out immediately after the activator solution had been added, and clotting time determined.

acidified solutions were even greater than those of the solutions without added acid. Balls, Thompson and Kies made a similar observation (5). They found bromelin solution kept at an acid pH and later

TABLE IV  
*Activation of Gelatin and Peptone Splitting Action of Papain with Different Activators*

Activator	Substrate	cc. $N/10$ NaOH
Thiosulfate	Gelatin	0.82
		1.57
		1.98
HCN	Cysteine	2.08
		0.57
		—
Thiosulfate	Peptone	1.18
		1.30
		1.10

0.5 cc. of a 5% solution of purified papain were mixed with 0.5 cc. of 0.01  $M$  activator solution and kept for 1/2 hour at room temperature. Then 9 cc. of substrate solution (2% gelatin or peptone, respectively, adjusted to pH 6 with McIlvaine's buffer) were added. With 5 cc. of the final mixture formol titration was performed while the rest was incubated at 40°C. for 6 hours. After this time formol titration was again performed. The difference between the two values so obtained indicate the proteolytic activity and are given above.

neutralized was more active in coagulating milk than the same solution which had not been acidified previously.

Table IV summarizes the results of some experiments which prove that the gelatin and peptone splitting actions are also activated by sodium thiosulfate.

The plant proteases of the papain group usually occur in the plant juices together with a natural activator (11). This activator is believed

TABLE V  
*Influence of Different Activators on the Gelatin and Peptone Splitting Activity of Fresh Ficus Latex*

Activator	Substrate	cc. N/10 NaOH
—		2.52
Thiosulfate	Gelatin	1.62
HCN		2.57
Cysteine		2.35
—		0.65
Thiosulfate	Peptone	1.28
HCN		0.53
Cysteine		0.59

Experimental procedure was the same as described in Table IV. Instead of ferment solution, crude ficus latex was used.

to be glutathione. The presence of such an activator is evident, for cysteine does not activate ficin in fresh ficus latex. If sodium thiosulfate is added to such a fresh ficus sap, the gelatin splitting action of the ficin is inhibited while the peptone splitting action is activated.

The milk clotting action of ficin in fresh ficus latex is equally inhibited by thiosulfate. In this case, it can be shown that the inhibition is a time reaction.

TABLE VI  
*Milk Clotting Action of Fresh Ficus Latex Diluted 1:10 with and without Added Activators*

Activator	Clotting Time minutes	Determination
—	0.56	—
Thiosulfate	0.56	immediately
Cysteine	0.56	immediately
Thiosulfate	1.50	incubated 3 minutes
Cysteine	0.66	incubated 3 minutes

## DISCUSSION

Sodium thiosulfate activated all six studied papainase preparations in their gelatin splitting, peptone splitting, and milk clotting activity. A more detailed study of this activation gave some results which may be discussed briefly in respect to the mechanism of the papain activation.

Bersin and Steudel (12) have shown that the reduction of dithiol compounds with -SH compounds only attains equilibrium after several hours. Even when a high potential difference exists between ferment and activator one would, therefore, expect activation to be neither instantaneous nor quantitative. We have shown that the activation of ficin and papain with regard to their milk clotting activity was almost instantaneous. It has been proved that this is a time reaction, although with difficulty because of the high speed with which the reaction occurs.

The reductive splitting of S-S groups is described in the literature as strictly dependent upon the pH. Bersin and Steudel studied the reduction of dithioglycolic acid with cysteine and found a decrease of the reaction velocity with decreasing pH (12). Ryklan and Schmidt determined the oxidation potentials of a number of organic thiol compounds in a solution of their reduced and oxidized form and found them to increase with decreasing pH (9). Slotta and Fraenkel-Conrat inactivated snake poison with cysteine, a reaction which they believe due to the reductive breakdown of an S-S linkage in the protein molecule. This reaction did not occur in acid medium (10). The activation of papain and ficin, however, did take place in dilute acid. A decrease of the velocity of the activation was not detectable.

Thiosulfate does not reduce S-S bonds at a significant rate. When inactivated papain preparations, which did not give a positive nitroprusside test, were activated with thiosulfate the nitroprusside test remained negative. These facts can not be easily explained by the old theory of the activation of papain, which assumes the reduction of an S-S group.

Fruton and Bergmann discussed another hypothesis for papain activation (13). They believe that papain is a dualistic enzyme consisting of an apoenzyme and coenzyme. Activation, according to this theory, consists of the replacement of an oxidized active group by an activated one. The activation would involve the formation of a new

dissociable compound between ferment and activator. The authors based this theory on the observation that papain and cathepsin may be inactivated by dialysis or precipitation with butyl alcohol, and reactivated with cysteine, and also the HCN or  $H_2S$  activated enzyme preparations may be inactivated by removing these activators by evacuation.

Our results above cited can best be explained by Bergmann's theory. Likewise, our observation that thiosulfate activates the peptone splitting action of ficin in fresh ficus latex while, under the same conditions, its gelatin splitting and milk clotting actions are inhibited, can best be explained by the coenzyme theory. One must suppose that ficin, plus natural activator as an active group, is the more active in splitting gelatin and clotting milk, while the ficin-thiosulfate compound is the more strongly active in splitting peptone. Moreover, the observation that equimolar amounts of thiosulfate activate approximately equal amounts of papain, and the fact that activation takes place in an acid medium, best fit the coenzyme hypothesis of papain activation.

It must be mentioned, however, that during an experiment in which fresh ficus latex was dialyzed for 3 days against distilled water, a product was obtained which was still somewhat inhibited in its milk clotting action by thiosulfate. This may have been due to the crude method used because of lack of suitable dialysis equipment, although the dialyzed preparation gave no reaction with sodium nitroprusside.

Recently, the coenzyme theory of papain activation has been questioned. Winnick, Cone, and Greenberg did not observe a substantial loss of activity of ficin by dialysis when anaerobic conditions were secured (14). But, while Bergmann had used crystallized dipeptides as substrate to check the ferment activity, these authors worked with the substrate casein. Scott and Sandstrom studied the relationship existing between activator concentration and ferment activity, obtaining results which are opposed to ours (8). They found that maximum activation is obtained by certain excess amounts of activators. They concluded that papain activation may be a surface phenomenon. They used the substrate gelatin. From a comparison of these observations, it seems likely that results obtained with different substrates cannot be compared. The coenzyme theory would explain a substrate specificity of papain preparations activated with various activators as explained above. Such a specificity has been supposed by Fruton and Bergmann (13). But in view of the discrepancy in the results of the

different authors the possibility must be kept in mind that the activation mechanism of the various substrate specific activities of papain may be a distinct one. This may be due to the fact that papain is a mixture of different enzymes, or that it possesses various active centers which are activated in a different way.

### SUMMARY

Sodium thiosulfate activates the gelatine splitting, peptone splitting and milk clotting action of all 6 papainases checked.

The activation of the milk clotting action is a time reaction.

Approximately equimolar amounts of thiosulfate and ficin or papain react to form an activated ferment.

The activation of the milk clotting action will take place in an acid medium.

The gelatin splitting and milk clotting action of fresh ficus latex is inhibited by thiosulfate while its peptone splitting activity is stimulated.

The possibility of a different activation mechanism for the activity toward distinct substrates is discussed.

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# The Effect of Tocopherol and of Fat on the Resistance of Rats to Anoxic Anoxia<sup>1</sup>

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Received July 16, 1945

## INTRODUCTION

In 1938 Campbell (1) discovered that rats maintained on a diet of carrots would resist anoxia to a considerable degree. As carrots are a rich source of carotene, Campbell was led to examine whether vitamin A or carotene administered with a non-carrot diet would duplicate the effect; they did not (2). In this laboratory it had been noticed that vitamin A is a specific and powerful catalyst for the oxidation of carotene. It was thought that the vitamin A from the carotene ingested by the rats in Campbell's experiment might be facilitating the use of oxygen under conditions of anoxia. Campbell had not described the doses of vitamin A and carotene used to imitate the carrot diet. Accordingly, we decided to repeat the experiments in the belief that the effect might be duplicated by massive doses of vitamin A and repressed or reversed by vitamin E. A preliminary experiment showed that the opposite was the case. Resistance to anoxia was decreased by vitamin A and to a lesser extent by carotene but was increased noticeably by vitamin E. Further experiments showed that selected diets supplemented with vitamin E enabled the rat and the guinea pig to withstand lower pressure and hence higher altitudes than usual.

## EXPERIMENTAL

The method of investigation was to place animals under a large glass bell jar which rested on a rubber gasket on a steel vacuum table. The pressure was reduced gradually according to an exactly reproducible schedule and the condition of the animals and their ultimate demise noted with the progress of time. The vacuum

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<sup>1</sup> Communication number 76 from the Laboratories of Distillation Products, Inc.

was produced and measured by a "high vac" pump and mercury manometer connected to the steel plate. The rate and degree of evacuation were controlled by leakage through a battery of four capillary orifices attached to the glass dome. Within the dome was housed a special wire mesh cage with four compartments, each large enough to hold two 300 g. rats comfortably. The entire apparatus was kept in an air-conditioned room at 26°C. A reproducible rate of decompression was obtained by following a standard pattern of manipulation of the four capillary tube pinch clamps. A typical curve showing rate of decompression appears in Fig. 1.

The experimental animals were placed in this chamber and the pressure gradually reduced for 15 minutes to about 185 mm. Hg. Survival time of the animals is measured from the end of this decompression period until death, indicated by cessation of breathing. The last few minutes of life were characterized by marked gasping.

The rats used in this study were normal male albino adults weighing from 250 to 320 g. and previously kept on a diet of Purina Dog Chow. Before the determinations of survival time under conditions of anoxia, groups of the rats were placed either on a stock diet or on one of the following diets deficient in vitamin E:

	Diet A per cent	Diet B per cent	Diet C per cent
Casein	20	20	20
Corn Starch	68	63	56
Salt Mixture	4	4	4
Brewers' Yeast	8	8	8
Lard	0	5	12

The lard was fortified with a vitamin A concentrate to give 10 units/g. of ration.

The rats were kept on these diets for two to three weeks, and during this time also received various tocopherol supplements, as indicated in the tables.

In presenting the results, average survival times were calculated for all experimental groups from percentage variation rather than actual time values. In every run, variations of each group from the response shown by the control group were calculated. These percentage variations were then averaged in each experiment. This procedure is believed to give a truer picture of the results than would simple arithmetical averages of the minutes survived of each group for all runs, since very slight variations in the conditions of decompression are reflected in quite large variations in the control groups' survival times from run to run.

## RESULTS

In preliminary experiments male guinea pigs weighing between 900 and 1100 g. were injected with 80 mg. of *d*- $\alpha$ -tocopheryl phosphate 18 hours before making the experimental decompressions. As is shown

in Table I, this treatment resulted in a substantial increase in the average survival time. However, when male rats weighing about 300 g. were similarly injected with 20 mg. of the tocopheryl phosphate, or fed single oral doses of mixed tocopherols 4 hours or 16 hours before experimental decompression, no beneficial effects were evident. These rats had been on the stock diet, containing ample vitamin E.

Adult male rats were then placed upon vitamin E low diets with various supplements. The data given in Table II indicate that on a

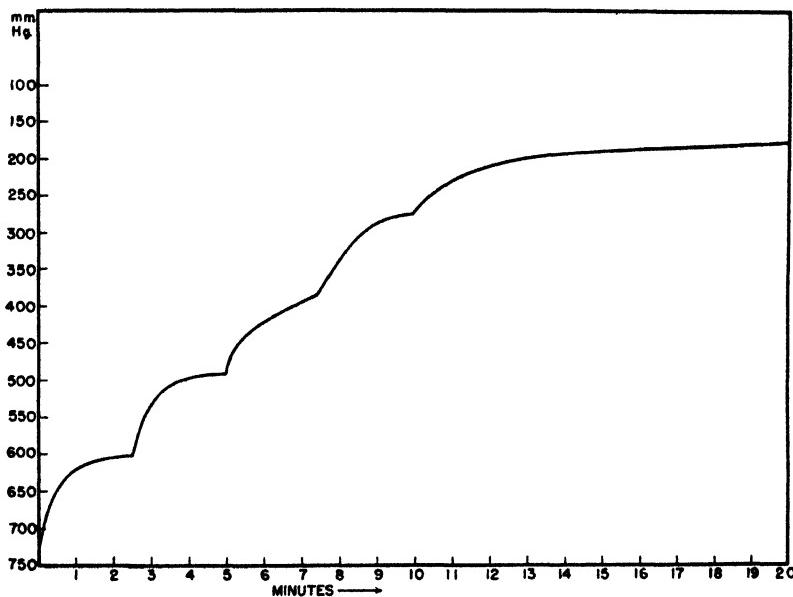


FIG. 1  
Curve Showing Standardized Rate of Decompression

diet which is also low in fat, vitamin E administration in daily oral doses effects a considerable prolongation in survival time. Moderate doses of carotene have little or no effect. On the diets low in vitamin E but containing fat (lard) the survival times of the controls were considerably reduced as compared with the controls on the low fat diet. Also, the beneficial action of the daily tocopherol administration is evident at much lower levels, and decreases as higher levels are given. Addition of the mixed natural tocopherol concentrate to the

diet at 15 mg. per cent, or an equivalent of 1.5 mg. of the mixed tocopherol per 10 g. of diet, results in no beneficial action. This may be related to the rapid destruction of the tocopherol when mixed into

TABLE I

*Preliminary Experiments on the Effect of Tocopherol on Survival Times of Guinea Pigs and Adult Rats Subjected to Low Atmospheric Pressures*

	Number of Runs	Number of Animals	Treatment	Average Survival Time of Controls	Average Deviation from Controls within Runs
Guinea pigs on rabbit chow	3	3	None	51.0	—
		3	Injection of 80 mg. $\alpha$ -tocopheryl phosphate 18 hrs. before decompression	—	+330
Male rats on stock diet	2	4	None	24.0	—
		4	Injection of 20 mg. $\alpha$ -tocopheryl phosphate 18 hrs. before decompression	—	-21
Male rats on stock diet	3	6	None	23.3	—
		6	20 mg. mixed tocopherols orally 16 hrs. before decompression	—	+7
Male rats on stock diet	4	7	None	32.3	—
		7	25 mg. mixed tocopherols orally 4 hrs. before decompression	—	+8

a synthetic diet. Rats kept on the stock diet and fed extra vitamin E daily again showed no significant change in survival time.

In another experiment forty-eight normal adult males were divided into two equal groups and placed on Diet A (low in fat) and Diet C (12% lard). Half of the rats on each diet were supplemented daily with 2.5 mg. of mixed natural tocopherols dissolved in 50 mg. of olive oil. After two weeks on this regimen the survival times under the standardized conditions of anoxia were determined. The results given in Table III show the survival time for the four groups of rats. In every determinative decompression two rats from each of the four groups were used and the average survival times of the pairs noted.

Since the average survival time for each group varied so considerably between runs, it was necessary to convert these responses to some common basis as shown in the lower portion of Table III. The survival

TABLE II

*The Effect of Tocopherols on Survival Times of Adult Rats Subjected to Low Atmospheric Pressure (185 mm. Hg. at 26°C.)*

Diet*	Number of Runs	Number of Rats	Daily Supplements*	Average Survival Time of Controls	Average Deviation from Controls within Runs
A. Low in Fat, A and E	8	8	None	34.4	per cent
			0.1 mg. carotene		+27
			3.0 mg. mixed tocopherols		+211
			carotene + tocopherols		+80
B. 5% lard, E low	4	10	None	13.3	+136
		10	0.5 mg. mixed tocopherols		
C. 12% lard, E low	3	3	None	14.0	+78 +29 +7 +6
		3	0.3 mg. mixed tocopherols		
		3	1.0 mg. mixed tocopherols		
		3	3.0 mg. mixed tocopherols		
		3	9.0 mg. mixed tocopherols		
C. 12% lard, E low	4	7	None	14.5	-13
		7	15 mg. % mixed tocopherols added to diet		
Stock Diet	2	5	None	27.2	+4 -20
		5	2.4 mg. mixed tocopherols		
		5	8.0 mg. mixed tocopherols		

\* The rats were given these diets and supplements for 15 to 20 days before decompression. They had previously been on a commercial dog chow diet. Unless otherwise indicated the supplements were fed by dropper, daily.

times of the rats maintained on the high fat diet and not receiving a tocopherol supplement were arbitrarily taken as 100 for each run and the other responses within runs were calculated proportionately. This part of Table III was then used to analyze the data statistically and

also to determine percentage variations in survival times due to dietary treatment.

In Table IV are tabulated the results of subjecting the data to an analysis of variance (3). It is apparent that in spite of the relatively great variations in survival times obtained in this experiment, the

TABLE III

*The Effect of Tocopherols and Fat on the Survival Times of Adult Male Rats Subjected to Low Atmospheric Pressure (185 mm. Hg. at 26°C.)*

## Ration and Supplement

Run	High Fat Diet		Low Fat Diet	
	- Tocopherol minutes	+ Tocopherol minutes	- Tocopherol minutes	+ Tocopherol minutes
1	11.5	25.0	26.0	38.5
2	18.0	18.5	45.5	22.5
3	33.5	42.0	31.0	110.0
4	35.0	22.5	26.5	39.0
5	19.5	23.5	26.5	65.5
6	27.5	36.5	25.5	33.5

<i>Relative Responses within Runs, Based on the Survival Time of the Group Fed a High Fat Diet without Tocopherol Supplementation, taken as 100</i>				
1	100	217	226	335
2	100	103	252	125
3	100	147	92	358
4	100	64	76	111
5	100	121	136	334
6	100	133	93	122
Totals	600	785	875	1385
		1385	2260	
Without tocopherol	1475			
With tocopherol	2170			
		3645—Grand Total		

differences in response between groups due to treatment are significant. The F value (5.00) calculated for the treatment variance is greater than the table value for F at the 5% point (3.10). If the F values had been the same it would have meant that there were only 5 chances in 100 that the differences in response between groups were due to chance and consequently were not significant. Since the F value for treatment

variance is actually larger the significance of the differences in response is greater than 95%.

Table IV also shows the component factors which comprise the treatment variance: fat in the diet, tocopherol supplementation, and interaction of fat and tocopherol. These factors are all significant at approximately the 5% point. In other words, fat in the diet influences the survival time irrespective of the tocopherol intake. Decreasing the fat in the diet from 12% to 0% increases survival time by 63%. Also, tocopherol supplementation is a significant factor regardless of the fat content of the diet. Increasing the tocopherol intake from zero to 2.5 mg. daily increased resistance to anoxia by 47%. However, the fact that the F value for the interaction variance was significant

TABLE IV  
*Analysis of Variance of Data in Table III*

Factor	Sum of Squares	Degrees of Freedom	Variance	F (by calculation)	F for P = 0.05
Total	170,489	23			
Treatment	73,093	3	24,364	5.00	3.10
Fat in diet	31,901	1	31,901	6.53	4.35
Tocopherol supplement	20,126	1	20,126	4.14	4.35
Interaction (Fat × tocopherol)	21,066	1	21,066	4.33	4.35
Error	97,396	20	4,870		

means that there was a definite interaction between the two factors, fat level in the diet and tocopherol supplement. These factors did not influence resistance to anoxia independently. Referring again to the percentage differences in response between the groups which can be calculated from the second part of Table III, it is evident that ingested tocopherol is most effective in increasing resistance to anoxia when the level of fat in the diet is low. For example, tocopherol increased survival time by about 31% on the high fat diet and 89% on the low fat diet. Conversely, decreasing the fat level of the diet from 12% to zero for the animals receiving tocopherol increased resistance by approximately 80%, while for the group without tocopherol supplementation the increase was only about 46%. Consequently it must be concluded that two interactions were in effect in this experiment: (1) Tocopherol decreases in effectiveness as the fat content of the

diet is increased. This, of course, may merely mean that the animals' requirement for tocopherols increases with increase in fat intake. (2) Also, decrease in fat intake is more effective in increasing resistance to anoxia when tocopherol is being ingested than on a tocopherol-low regime.

### DISCUSSION

The short survival time of rats on a vitamin E deficient diet, compared with that of rats receiving tocopherol supplements, indicates an earlier stage of vitamin E deficiency than had been evident from other functional abnormalities reported. The rats had been placed on the vitamin E deficient diet after reaching adulthood and were on that diet less than three weeks. The dog chow diet upon which they had been reared is probably low in vitamin E, yet it does have enough of the vitamin to support normal reproduction. The tocopherol concentration in the blood of a few rats which had been on the vitamin E low diet (Diet A) for 20 days was 0.15 mg. per 100 ml. of plasma, while in similar rats which had received 3 mg. of mixed tocopherols daily the plasma content was 1.0 mg. per 100 ml.<sup>2</sup> A vitamin E deficiency in the former group could be surmised from these chemical analyses.

The guinea pig is known to be much more dependent than the rat upon tocopherols to prevent acute somatic symptoms of vitamin E deficiency. In line with this, injections of single large doses of *d*- $\alpha$ -tocopheryl phosphate into guinea pigs were beneficial, while doses that were the same size on the basis of body weight had no effect on the survival time of rats.

The effect of tocopherols on the physiology of oxygen utilization may be related to Houchin's report (5) that tissues from vitamin E deficient animals had an abnormally high oxygen uptake in the Warburg apparatus and also *in vivo* (6). A correlative field for speculation is opened by the results of LeBlond (7), who showed that thyroidectomy greatly increased the resistance of rats to anoxic anoxia, while injections of thyroxine or dinitrophenol into normal or thyroidectomized rats lowered their resistance. Thiourea (7) and thiouracil (8) increase survival time of rats subjected to acute anoxia. These

<sup>2</sup> Tocopherol analyses (4) of these blood samples were kindly performed by Dr. M. L. Quaife.

are known to be B.M.R. depressants and are also water-soluble antioxidants.

The carrot diet effect in increasing resistance to anoxia possibly may be due to a high tocopherol and low fat combination. It is interesting that a carrot diet provides protection against carbon tetrachloride toxicity (9). The liver damage resulting from such toxicity is thought to be due to impairment of tissue respiration resulting in a type of anoxia. It was shown that the protective substance in carrots is extractable with 95% ethanol and is very unstable.

On a diet in which part of the carbohydrate is replaced by fat the animal would presumably have a greater oxygen requirement since the respiratory quotient would be lower. This lower R.Q. is reflected in the fact that the rats on the fat diets survived a shorter time than those on the low fat diets. At high levels of tocopherol a depressant effect on survival to anoxia has been noted. This may be correlated with the observations (10, 11) that the tocopherols at higher levels depart from their role as antioxidants and become prooxidant in action.

#### SUMMARY

1. Natural mixed tocopherols given orally in physiological quantities to adult rats result in a time of survival to anoxic anoxia greater (+ 89%) than that of controls on a vitamin E-free, low fat diet. The effectiveness of tocopherol is decreased (to about + 31%) when the diet contains fat at a 12% level.
2. Fat in the diet decreases survival time of rats under conditions of anoxia, possibly by decreasing the animals' R.Q.
3. Injection of large single doses of *d*- $\alpha$ -tocopheryl phosphate greatly increases the time of survival to anoxia of guinea pigs on a rabbit chow diet, but has no effect on rats.

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# **Factors Influencing the Destructive Effects of Acidic Beverages on the Teeth of White Rats and Hamsters \***

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Received September 4, 1945

## **INTRODUCTION**

Tooth erosion is a rather commonly observed but little understood phenomenon. In a few instances (1, 2, 3, 4) it has been pointed out that the acid in certain fruit juices, soft drinks and hard candies may be destructive to human teeth; the present authors have recently observed one instance of very severe damage to the teeth of a 32 year old man apparently resulting from the practice of sucking the juice from two or more oranges per day over a period of two years. Because the erosion from acids usually occurs over the entire tooth surface, it normally escapes detection *in vivo* except in severe cases. Mild effects, which would pass unobserved in the mouth, might be found in examining extracted teeth.

This has been further indicated by experiments with monkeys, details of which will be reported later. After drinking 170 ml. of cola beverage or synthetic lemonade daily for one month, distinct decalcification of the teeth was readily noted only after they were extracted and examined.

In an earlier report (5) we presented the destructive effects on the teeth of rats and puppies caused by consuming limited amounts of a popular soft drink over periods ranging from one week to several months. The low pH (2.6) imparted to this beverage by the phosphoric acid component was the primary factor in etching the enamel, but it was further demonstrated that the presence of sucrose increased the destruc-

\* The opinions and views set forth in this article are those of the writers and are not to be considered as reflecting the policies of the Navy Department.

tive effects of the phosphoric acid solution. The inclusion in the solutions of 1 to 20 ppm. of fluorine (as NaF) was found to decrease the etching of the enamel.

The present study was carried out in order to (a) ascertain the effects of beverages containing acids other than phosphoric, (b) study the influence of sucrose on *in vivo* tooth decalcification by these other acids, (c) determine the ability of sugars other than sucrose to increase the destructive action of phosphoric acid on teeth, and (d) observe whether the various acid solutions, both with and without sucrose or fluoride, behave the same on pulverized enamel *in vitro* as they do *in vivo*. Because the molars of the hamster are structurally more analogous to human teeth than are those of the rat, this species was used to check some of the results with fluorine previously obtained on the rat.

#### PROCEDURE

*Animals.* The white rats used in these studies were from six to seven weeks of age and weighed about 100 g. at the beginning of the experiment. The hamsters were young animals of unknown age and ranged in weight from 50 to 100 g., with an average of about 70 g. All were kept in individual cages so that the food \* and fluid intake could be regulated. In any given experiment the animals were grouped as evenly as possible according to age (or litter), weight and sex. At the completion of each experimental period the animals were sacrificed and their jaws removed, preserved and graded according to the procedure described previously (5, 6). According to this system each molar, particularly the lingual surface, is carefully examined under a dissection microscope and assigned a score ranging from 0 to 6, depending on the extent of destruction to the enamel and dentin.

*Solutions.* The solutions employed, all of which had a pH of 2.5 to 2.6, contained (a) phosphoric acid, 0.055%, (b) sulfuric acid, 0.024%, (c) citric acid, 0.20%, or (d) lactic acid, 0.43%. Either sucrose (10%) or saccharin (37.5 mg. %) was added to each to give the desired sweetness. Navy-issue synthetic lemonade was used in a few instances.

In one experiment equivalent amounts of maltose, glucose or lactose were used in place of sucrose in the phosphoric acid beverages. As the sweetness of these sugars is less than that of sucrose (7), variable amounts of saccharin were added to bring the solutions up to the sweetness of 10% sucrose.

In the hamster studies, sodium fluoride was added to some of the solutions to give levels of 1, 5, 10, and 20 ppm. of fluorine.

All experiments, except where otherwise stated, were of one week duration, during which time the animals received 20 ml. daily of the acid beverage, 10 ml. in the morning and 10 ml. in the afternoon.

*In vitro experiments.* *In vitro* studies of the decalcifying properties of most of the

\* G.L.F. dog feed from Canandaigua, N. Y.

above-mentioned solutions were carried out in triplicate as follows: A 10 mg. sample of dry, powdered, human enamel (100-mesh) was weighed into a glass-stoppered 25 ml. erlenmeyer flask. Ten ml. of the appropriate acid solution were then added, the timing being started as the pipette was opened. After twenty seconds the pipette was removed, the stopper inserted and the flask shaken by violent wrist action. At the end of two minutes the mixture was poured onto a filter with a slight vacuum sufficient to give complete filtration in fifteen to twenty seconds.

A 2 ml. aliquot of the filtrate was adjusted with dilute ammonium hydroxide to about pH 5.9 (using brom-cresol purple), and 2 ml. of 4% ammonium oxalate were added. After standing overnight the tube was centrifuged, the supernatant liquid siphoned off, and the residue washed twice with a mixture of 3 ml. of 0.6% ammonium hydroxide and 1 ml. of acetone. The precipitated calcium oxalate was then dissolved in 1 ml. of normal sulfuric acid and titrated from a microburette with 0.0025 N potassium permanganate solution. Reagent blanks, in duplicate, were run for each analysis.

## RESULTS AND DISCUSSION

### *Rat Studies*

(a) *Comparative effects of various acids.* The *in vivo* effects of different acids on the molars of rats disclosed some striking differences (Fig. 1). The average tooth scores obtained with groups of fifteen or more animals consuming various sweetened acid solutions were as follows: lactic acid, 3.6; phosphoric acid, 3.5; citric acid, 2.7; and sulfuric acid, 2.7. But these scores alone give an incomplete picture of the action of the different acids; it is necessary to compare the relative effects on the upper and lower molars separately. Thus, it is evident that lactic and citric acids caused marked damage to the lower molars (average scores of 5.2 and 3.9, respectively), but in most instances had a relatively mild effect on the uppers (average scores of 2.0 and 1.4, respectively). This mild effect on the upper molars was more consistently obtained with the rats on citric acid. Sulfuric acid, on the other hand, had a moderate effect on the lowers (average, 2.4), with somewhat greater destruction of the uppers (average, 2.9). The action of phosphoric acid-sucrose was comparable to that of citric acid on the lower molars and to that of sulfuric acid on the uppers.

The surprising results obtained with the 0.2% citric acid solution were confirmed with a beverage prepared from a synthetic lemon juice powder in common use by the Army and Navy. This product contains the following percentages of various constituents: lemon juice solids, 8.5; anhydrous citric acid, 33.9; corn syrup, 41.4; glucose, 14.5; oil of lemon, 0.3; tricalcium phosphate, 0.5; and ascorbic acid, 0.9. When

made up according to directions on the can, the lemonade has a pH of 2.6. Four rats drinking this beverage for one week had average molar scores of 5.7 and 1.0 for the lower and upper molars, respectively; others maintained on the lemonade for 30 days showed similar effects except that the mandibular molars were dissolved away nearly to the gum line (Fig. 2). After five months on this beverage the lower molars were sometimes completely destroyed beyond the level of bifurcation of the roots, so that only isolated roots remained.

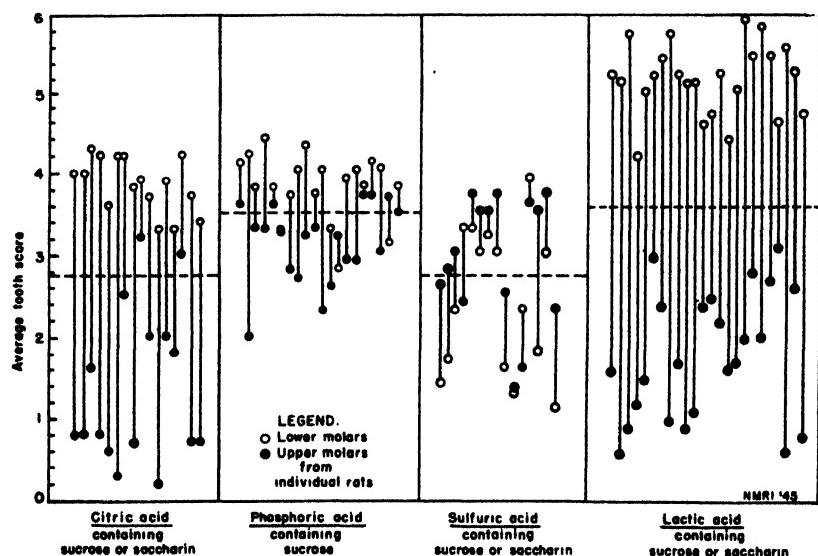


FIG. 1

*In Vivo Effects of Different Acid Solutions on the Molars of Rats*  
The broken lines indicate the average tooth score for both jaws. Corresponding jaws from a given animal are lined up vertically.

McClure (2) observed that acid beverages seem to attack the lower molars of rats to a greater degree than the uppers. To our knowledge, however, no one has previously reported the peculiar effects of different acids described here. The results were consistent in all experiments, most of which were repeated three or four times.

(b) *The significance of sugar in tooth destruction by acid beverages.* It was reported earlier (5) that when saccharin was substituted for sucrose in a sweetened phosphoric acid solution, much less damage resulted to

the enamel of rats drinking this solution. Viscosity was not an important factor in the different action of the two solutions.

The replacement of sucrose by saccharin in solutions of lactic, citric and sulfuric acids did not, however, influence the extent to which these acids attack the teeth. Using from eight to twenty rats in each group the following average molar scores were obtained: phosphoric acid-saccharin, 1.4; phosphoric acid-sucrose, 3.5; lactic acid-saccharin, 3.7; lactic acid-sucrose, 3.4; citric acid-saccharin, 2.7; citric acid-sucrose, 2.6; sulfuric acid-saccharin, 2.4; sulfuric acid-sucrose, 2.9.



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FIG. 2

Lingual View of Upper and Lower Molars from a Rat Which Had Drunk Synthetic Lemonade for 30 Days

The upper molars are essentially unaffected; the lower molars show extreme destruction of enamel and dentin above the gum line. The broken lines indicate the approximate normal contours of the lower molars.

When other sugars were incorporated in place of sucrose in the phosphoric acid beverage, it was observed (Fig. 3) that glucose and maltose behaved as did sucrose, whereas lactose did not. As the rats receiving the acid-lactose refused to consume 20 ml. daily of the solution (they averaged about 17 ml.), a paired-drinking experiment was conducted which confirmed the difference between lactose and the other sugars studied.

A possible working hypothesis to explain the greater decalcification by phosphoric acid in the presence of certain sugars is that glucose may be phosphorylated in the mouth and split to form acid degradation

products which also attack the teeth. This hypothesis would explain why sucrose acted differently with phosphoric acid than with the other acids. It would also account for the inability of lactose to accentuate the decalcifying properties of a phosphoric acid solution, since lactose is stable in acid and the enzyme lactase is absent from the saliva. Sucrose, on the other hand, is readily inverted at pH 2.6, and maltose may be appreciably split to glucose by the salivary maltase. The actual mechanism involved is being investigated.

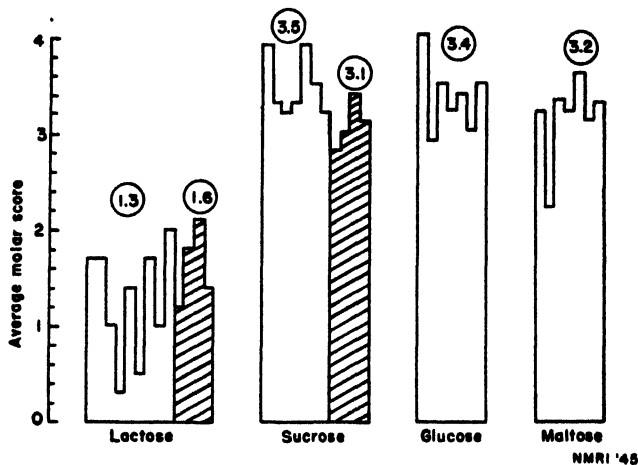


FIG. 3

Effects of Different Sugars on *In Vivo* Tooth Decalcification by Phosphoric Acid (pH 2.6)

The shaded portions represent litter mates drinking lactose and sucrose in a paired-drinking experiment. Each vertical bar corresponds to one rat; the numbers above each group give the average molar score for that group.

**Hamster studies.** The Syrian hamster is a small rodent which differs from the rat in that its molars are completely covered by enamel in much the same manner as human teeth. For this reason it was chosen for further experimentation on the effects of small amounts of soluble fluoride ( $\text{NaF}$ ) in counteracting enamel decalcification by acid. It was soon ascertained that the hamster does not have as great a craving for the citric acid-sucrose solution as the rat; accordingly, instead of drinking 20 ml. daily of the experimental solutions, the average consumption by the hamster was about 10 ml. per day.

The results, as summarized in Fig. 4, confirm the findings obtained previously with the rat and dog (5). In about 75% of the animals, fluorine afforded partial protection to molars exposed to a citric acid solution for one week. The data suggest that fluorine was more effective at the higher levels, but this cannot be concluded because of the limited number of animals employed. It should be noted, also, that the effect of

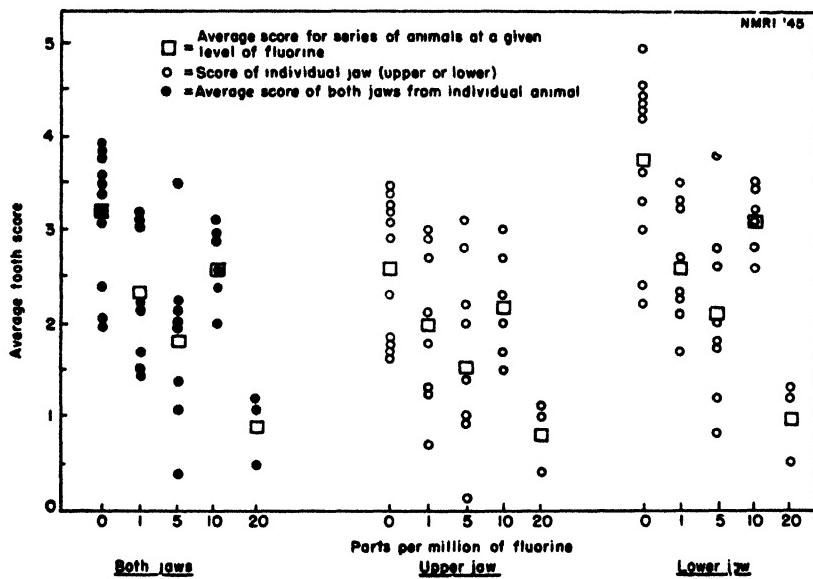


FIG. 4

Influence of Different Levels of Fluorine (as NaF) in Counteracting Tooth Destruction of Hamsters Consuming a 0.2% Citric Acid-10% Sucrose Solution for One Week

citric acid on the upper molars of hamsters was very different from that found with the rat (Fig. 1).

In one instance the phosphoric acid-sucrose solution was given to four hamsters over a two-week period, during which time the average daily consumption was about 18 ml. Subsequent examination disclosed severe damage to both the upper and lower molars (average tooth scores 3.8 and 4.7, respectively), comparable to what had been obtained with the rat.

*In vitro studies on enamel solubility.* The data in Table I indicate that

the effects obtained on pulverized human enamel *in vitro* differ in several respects from the *in vivo* results. Good check results were obtained on a given solution on different occasions.

Of the three acids tested, citric acid was found to decalcify the enamel to the greatest degree, followed by phosphoric and sulfuric acids, respectively. This observation is at variance with our *in vivo* results and with *in vitro* studies of Thurlow and Bunzell (8), who reported that enamel was progressively less soluble in phosphoric, citric and sulfuric

TABLE I

*In Vitro Solubility of Powdered Human Enamel in Various Acid Solutions (pH 2.6)  
With and Without Fluorine*

Solution	Amount of calcium dissolved*			Difference mg.	per cent
	No F present mg.	6 ppm. F present mg.			
Water (control)	0.07	—			
<i>Phosphoric acid</i>					
alone	1.47	1.02		0.45	30
plus saccharin	1.63, 1.67	1.40		0.25	15
plus sucrose	1.31, 1.35	1.13		0.20	15
<i>Sulfuric acid</i>					
alone	1.39, 1.40	0.99		0.41	29
plus saccharin	1.16				
plus sucrose	1.02				
<i>Citric acid</i>					
alone	1.85				
plus saccharin	1.80	1.55		0.25	16
plus sucrose	1.58	1.33		0.25	14

\* See text for procedure. Each value stated represents an average of duplicate or triplicate analyses run simultaneously. Agreement between analyses was within 5%.

acids. It does, however, confirm their contention that, at a given pH, enamel solubility is influenced by the anion of the acid used.

When sucrose or saccharin was added to the different acid solutions the enamel solubility was altered somewhat, but not in accordance with the effects noted *in vivo*. In every instance of the *in vitro* studies, the acid-sucrose solution was less decalcifying than either the corresponding acid alone or the acid-saccharin solution. The inclusion of 6 ppm. fluorine in the solutions, however, invariably lowered the enamel

solubility by 14 to 30%, an observation consistent with that found *in vivo*. Others (9, 10) have previously observed, *in vitro*, the lessened solubility of enamel in the presence of fluorides.

#### ACKNOWLEDGMENT

The assistance of the following persons, in the care of animals and preparation of the jaws, is gratefully acknowledged: E. M. Osborne, E. L. Lee and J. E. Horlander.

#### SUMMARY

1. The effects on the molars of rats drinking solutions of citric, lactic, phosphoric and sulfuric acids were studied. At the same pH (2.6), the various acids attacked the teeth in differing degrees.
2. Citric and lactic acid solutions, in contrast with the action of the other two acids studied, caused severe damage to the lower molars of rats but, in most cases, had relatively much less effect on the uppers. Comparable results were noted when Navy-issue synthetic lemonade was used.
3. The presence of 10% sucrose, glucose or maltose increased the tooth-destructive properties of phosphoric acid solutions *in vivo*. Lactose, on the contrary, had no such effect. Citric, lactic and sulfuric acid solutions were not influenced in their decalcifying properties by the inclusion of 10% sucrose.
4. Experiments with hamsters receiving citric acid solutions containing 1, 5, 10, and 20 ppm. of fluorine (as sodium fluoride) produced results comparable with those reported previously for the rat. Fluorine provided, in most instances, partial protection against tooth destruction by acid. The markedly different action of citric acid *in vivo* on the upper versus lower molars of rats was not observed in the hamster.
5. Studies *in vitro* on the decalcification of pulverized human enamel by various acids, both alone and in the presence of sucrose or saccharin, yielded results differing in several respects from those noted *in vivo* with the rat. Whereas phosphoric acid-sucrose caused greater tooth destruction *in vivo* than did citric acid, this situation was reversed *in vitro*. Also, *in vitro* sucrose failed to increase the decalcification of enamel by phosphoric acid. When fluoride was added to the acid solutions, however, the enamel solubility *in vitro* was lowered 14 to 30%.

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# **Preparation of the Egg Yolk Lipoprotein, Lipovitellin**

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Received September 10, 1945

The preparation and properties of the lipoprotein of egg yolk, lipovitellin, has been reported by Chargaff and others (1, 2, 3, 4). Chargaff's method of preparation consists of diluting emulsified yolk with an equal volume of saturated NaCl solution and extracting with ether. The salt solution is dialyzed and the precipitated lipoprotein collected by centrifugation. This procedure is repeated and the final preparation emulsified in water, frozen by immersion in CO<sub>2</sub>-alcohol, and dried from the frozen state. Such preparations contain about 18% of phosphatides, which can be extracted with alcohol but not with ether. The alcohol removes about 5% of fats other than phosphatides and decomposes the lipoprotein as it frees the combined lipid.

In work in this Laboratory on identification of deteriorative chemical changes in stored dehydrated whole egg and yolk, it was desirable to separate the yolk into its components without the use of organic solvents. On passage of emulsified egg yolk through a Sharples centrifuge it was found that most of the lipovitellin was deposited in the bowl as a waxy material largely soluble in 10% NaCl. This crude lipoprotein was used for studies in which palatability determinations were essential. However, further purified lipovitellin could readily be prepared by extraction with ether, solution in salt solution and precipitation by dialysis. Since the method avoids ether extraction of egg yolk diluted with salt solution, which often results in troublesome emulsions, it offers marked advantages over published methods for the isolation of this protein and is reported here together with some analyses of the product.

## **EXPERIMENTAL**

Fresh eggs were obtained from wholesale dealers. The yolks were prepared by separation from whites and careful washing with water

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before the yolk membrane was broken and the contents collected. Preliminary experiments showed that recovery of the lipoprotein was more nearly complete if the yolk was first diluted with two volumes of distilled water before centrifuging. The diluted emulsified yolk was then centrifuged in a Sharples supercentrifuge; the lipoprotein was deposited as a waxy translucent solid while most of the fat and the water-soluble protein were thrown off as an aqueous emulsion. The precipitated

TABLE I  
*Composition of Lipovitellin and Vitellin \**

Sample	Alcohol soluble fraction of lipovitellin	Per cent Nitrogen in:			Per cent Phosphorus in:			Per cent Ash in:	
		Lipo-vitellin	Vitellin	Phospho-lipid	Lipo-vitellin	Vitellin	Phospho-lipid	Lipo-vitellin	Vitellin
<i>per cent</i>									
1	17.6	12.7	15.01	1.59	2.34	2.13	3.28	—	6.62
2	16.2	13.0	15.02	1.69	2.34	2.11	3.27	—	—
3	18.5	12.8	15.35	1.54	2.32	2.13	3.26	—	7.12
4	15.4	13.0	—	1.57	2.37	2.01	3.29	—	—
5	16.2	13.0	—	1.69	2.33	2.06	3.28	—	—
6	16.2	12.8	—	1.4	2.33	2.09	3.28	—	—
7	18.5	13.07	15.61	—	1.47	1.06	—	3.80	3.32
8	17.7	13.01	15.61	—	1.56	1.16	3.28	4.38	3.89
9	16.1	13.18	15.65	—	1.48	1.01	—	3.53	3.31
10	16.5	13.02	15.62	—	1.52	1.12	—	4.26	3.57
11	16.7	13.08	15.60	—	1.51	1.10	—	4.04	3.40
12	15.7	12.8	15.20	1.55	—	—	3.30	—	—
13	—	12.95	—	—	1.40	—	—	0.94	—
14	—	13.05	—	—	1.30	—	—	1.38	—

\* All results on dry-weight basis.

material contained approximately 90% of the total lipovitellin present in the yolk, as determined by isolation of the remainder by ether extraction of the lyophilized Sharples supernatant, extraction of the residue with 10% NaCl and dialysis to precipitate the lipovitellin.

Chargaff (1) has shown that ether does not break up the protein-lipid complex of yolk protein; the lipoprotein can therefore be freed of uncombined fat by ether extraction. Removal of the free fat was carried out in two ways. With some samples the product was emulsified in water and dried from the frozen state before extraction with ether

(Samples 1 to 6, Table I), while for others the material was extracted directly at reduced temperature in the moist form by agitation and centrifugation (Samples 7 to 11, Table I). Approximately 15–16% of the total material was extractable with this solvent, leaving the lipovitellin free of uncombined fat. The product after extraction was soluble in salt solution, being most readily soluble at salt concentrations between 5 and 10%. The yields of lipovitellin obtained from eight Sharples precipitates varied between 15.5 and 17.4% of the egg yolk solids, with an average yield of 16.6%.<sup>2</sup>

Alcohol extraction of the ether-insoluble product removed an amount of material equal to an average of 16.8% of the total. The insoluble fraction (vitellin) was now insoluble in water and salt but could be dissolved in alkali. At a pH of *ca.* 10.0 the solutions became very viscous and glue-like in character, very similar to alkaline solutions of casein. On acidification the material again precipitated.

Comparative analysis of lipovitellin prepared as described by centrifugation of diluted egg yolk followed by ether extraction and vitellin obtained by alcohol extraction of lipovitellin revealed phosphorus contents of the products to be higher than those published; for vitellin the values were approximately doubled. Phosphorus was determined by the method of Allen (5) and nitrogen by the Kjeldahl-Gunning-Arnold procedure (6). The ash content was likewise found to be high (samples 1 to 6). After the lipovitellin was taken up in 10% NaCl to a 10% solution and precipitated by dialysis three times, the values for phosphorus were reduced to approximately that previously reported by others, and the ash was likewise found to be reduced to approximately half that of the undialyzed samples (samples 7 to 11). The dialysis was carried out in rotating cellophane bags, at 4°C., for approximately 24 hours, against flowing distilled water which was constantly being deionized by passing over synthetic cationic and anionic resins. Analysis of the ash showed that 22.5% consisted of phosphorus, which accounts for 70% of the phosphorus found in the vitellin.<sup>3</sup> Only 5.2% of the ash was found to be calcium. Reprecipitation of two samples of lipovitellin

<sup>2</sup> The yield of lipovitellin reported here is very much lower than values given in the literature (7). This appears to be due to the presence in egg yolk of another lipoprotein which is not isolated with the lipovitellin when the present method is used. The properties of this material will be described in a later communication.

<sup>3</sup> The ash was determined by the straight ashing procedure. The values are therefore minimum values, since some P<sub>2</sub>O<sub>5</sub> was probably volatilized during the procedure.

from 10% NaCl six and twelve times by dialysis (samples 13 and 14, respectively) lowered the ash content to 0.94 and 1.38%.

Sample 12 (Table I) was prepared by dissolving the centrifuge precipitate in ten volumes of 10% NaCl solution, followed by dialysis to reprecipitate the protein. Analysis of the product after the first precipitation gave a value of 10.5% nitrogen. The material was redissolved and reprecipitated three times before the nitrogen value reached that of the samples prepared by ether extraction. No increase in nitrogen content resulted from further repetition of the process, and no material was extracted by ether after the nitrogen value had become constant.

The lipid extracted from the lipovitellin by alcohol was soluble in ether and from its appearance, ready emulsification in water and precipitation with acetone, it obviously consisted mainly of phospholipid. Phosphorus and nitrogen analyses of the material gave average values of 3.27 and 1.58%, respectively, or a ratio of phosphorus to nitrogen of 0.93. This ratio corresponds rather well with that for cephalin or lecithin, but the ready solubility of the material in cold alcohol indicates that it was at least mainly lecithin. The low values for phosphorus and nitrogen and the fact that some amino nitrogen was present indicate, however, that the phospholipid was not pure lecithin.

#### ACKNOWLEDGMENT

We are indebted to L. M. White, Geraldine Secor, C. M. Johnson, E. F. Potter and Bernice Morrison of this Laboratory for the nitrogen, phosphorus, moisture and ash determinations, and to Adele Lausten for assistance in some of the preparative phases.

#### SUMMARY

A convenient method for the preparation of the lipoprotein from egg yolk consists of collecting the crude lipoprotein by passing diluted egg yolk through a Sharples centrifuge and extracting the precipitate with cold ether. The product thus obtained contains, in addition to lipovitellin, phosphorus-containing substances, which are removed by solution in 10% NaCl and precipitation by dialysis. The analytical data for the final product agree well with published values.

Approximately 18% of egg yolk solids was found to be lipovitellin; roughly 17% was isolated by the reported method.

The lipoprotein contains approximately 16 to 18% of a phosphatide, which appears to be largely lecithin.

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## **Estimation of Blood in Tissue \***

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Received October 19, 1945

### **INTRODUCTION**

The estimation of blood in tissue is of some utility, *e.g.*, in the study of the distribution of any substance between blood and tissue. The methods that have been proposed are based on a comparison of the concentration of hemoglobin or its derivatives in blood and tissue, the concentration being estimated spectrophotometrically (1, 2). In these methods turbid extracts of tissue have been used. The absorption of light due to hemoglobin in such extracts has been obtained by correcting the measured light absorption for the effect of turbidity by assuming that the absorption of light due to turbidity increases linearly with decreasing wave length of incident light (1). The concentration of hemoglobin in such extracts has also been estimated in terms of the changes in light absorption produced by the conversion of methemoglobin to cyanmethemoglobin (2). This procedure avoids error due to the presence of methemoglobin in blood or tissue or both. It is assumed that the turbidity is not changed by the measures effecting the conversion of methemoglobin to cyanmethemoglobin.

The method given below is based on the observation of Michel *et al.* that turbid mixtures containing hemoglobin or its derivatives can be clarified by addition of ammonium sulfate to about half saturation followed by filtration. This procedure was used in the estimation of the relative concentrations of hemoglobin and derivatives in mixtures containing comparatively large amounts of total pigment (3, 4). The total hemoglobin in optically clear filtrates obtained by treatment of tissue extracts with ammonium sulfate can be estimated as cyan-

\* Aided by a grant from the Rockefeller Foundation.

methemoglobin by means of any suitable spectrophotometer. In the present work the Evelyn photoelectric colorimeter was used (5). The method avoids the assumptions inherent in the estimation of hemoglobin in turbid solutions and the instrumental errors involved in obtaining a correction for turbidity or in the two measurements required for estimation of the change of methemoglobin to cyanmethemoglobin.

### METHOD

The tissue is finely ground with water by any suitable procedure, *e.g.*, by the use of a blender, homogenizer, or in a mortar. Routinely, 10 ml. of water are used per g. of tissue. If inspection indicates that the final concentration of hemoglobin will be too high, more water should be used. After the addition of a few drops of toluene per 10 ml. of water, the mixture is allowed to stand for about 30 minutes with occasional mixing. While the tissue is standing, blood obtained from the animal at the same time the tissue was removed is diluted 1/100, 1/150, 1/200, 1/300 and 1/350 with water. These mixtures are allowed to stand 5 to 10 minutes. The dilutions of blood indicated are such that the colorimeter readings finally obtained will employ the useful range of the instrument. If the concentration of cells in the blood is unusually high or low, other dilutions may be used.

One volume of ammonium sulfate-phosphate solution, pH 6.6 (1 volume of 0.5 M dipotassium hydrogen phosphate plus 50 volumes of saturated ammonium sulfate), is then added per volume of water in each mixture of blood and tissue, the water in the tissue being included. For most purposes, it may be assumed that the tissue contains 75% water. After standing 10 to 20 minutes with occasional mixing, the mixtures are filtered through Whatman paper, No. 30, of a size that will just contain all of a given mixture. The volume of a given mixture should be such, *e.g.*, 30 ml., that about 12 ml. of filtrate are obtained within a reasonable period of time. By the use of the microcolorimeter, with which 2 ml. of filtrate are sufficient, the quantity of tissue required can be reduced considerably.

Ten ml. of a mixture of equal volumes of ammonium sulfate-phosphate solution and water which serves as a blank, and 10 ml. of each filtrate are transferred to colorimeter tubes. One drop of potassium ferricyanide solution, 20 g. per 100 ml., is added to each tube. After mixing one drop of potassium cyanide solution, 5 g. per 100 ml., is added to each tube. After mixing, the per cent transmission of each solution is measured using filter No. 540, the blank being set at 100% transmission. The values representing per cent transmission are converted into the values representing the corresponding optical densities. In the nomenclature introduced by Evelyn these values are designated respectively as *G* and *L*.

The optical densities which would obtain for undiluted blood and tissue are calculated. The average optical density for undiluted blood is calculated. The ratio of the calculated optical density of undiluted tissue to that of undiluted blood represents the concentration of blood

in the tissue, the units of concentration being ml. of blood per g. of tissue.

The following test indicates the precision obtainable with the method. Kidney was homogenized with water. Six identical and five different aliquots, the volumes of mixture to be treated with sulfate-phosphate mixture being made the same by addition of water, were assayed. The average concentration of blood found was 0.088 ml. per g. with a standard deviation of 0.002. Similar results were obtained with other tissues. In addition, a number of assays were carried out on brains using two samples of finely ground frozen tissue for each. The average difference between duplicates was 6%, the maximum, 15%. The maximum difference between duplicate assays of the same tissue preparation was 5%.

The extraction of blood from tissue seems reasonably complete. This is suggested by the following test. Samples of finely divided tissue were ground with amounts of water varying from 5 to 20 ml. per g. The resulting mixtures were treated in the usual way with the sulfate-phosphate solution. The maximum difference between optical densities corrected for dilution, was obtained with a sample of liver, in which case the optical density obtained with the most dilute preparation was 10% higher than that obtained with the most concentrated. The order of the differences in corrected optical density obtained for several different dilutions of a given tissue was usually the same as indicated for the sample of liver.

The following data indicate the extent of recovery of added blood. The average per cent recovery and standard deviation in the case of brain, heart, kidney, liver, lung, leg muscle, heart and spleen was  $96 \pm 9$ ,  $97 \pm 4$ ,  $108 \pm 8$ ,  $98 \pm 7$ ,  $96 \pm 5$ ,  $98 \pm 7$  and  $101 \pm 8$  respectively. The blood was added to initial tissue preparations in five different amounts that were sufficient to increase the initial concentrations of blood by a minimum of about 15% to a maximum of about 300%.

In most of the assays carried out by the method the change in optical density produced by the conversion of methemoglobin to cyanmethemoglobin was also measured. With all tissues employed the concentration of blood indicated by this measurement agreed satisfactorily with that obtained by measurement of cyanmethemoglobin alone. This indicates that no pigment appeared in the final solutions that did not have these properties of hemoglobin. Comparison of results obtained by both procedures provides a convenient way of testing for the presence of interfering pigment.

The method given has been compared with those in which turbid extracts are used (1, 2). With skeletal muscle, heart and lung, which yield comparatively clear extracts, and when the concentration of blood was greater than 0.05 ml. per g., the results agreed fairly well. With the other tissues, except when the concentration of blood was greater than 0.10 ml. per g., the agreement was not consistently satisfactory.

Representative data are given in Table I. The tissues and bloods were obtained from cats given a lethal dose of a barbiturate. It should be pointed out that no attempt has been made to exclude myohemoglobin in the case of skeletal muscle and heart. The results for these tissues are, therefore, too high.

TABLE I  
*Concentration of Blood in Tissues of the Cat*

Tissue	Concentration of Blood ml. per g.
Brain	0.030
Heart	0.084
Kidney	0.093
Liver	0.052
Lung	0.147
Skeletal muscle	0.027
Spleen	0.195

The data given are averages obtained with at least two animals.

The technical assistance of Miss Ruth Hurwitz is gratefully acknowledged.

### SUMMARY

A method for the estimation of blood in tissue is presented. The method is based on the spectrophotometric determination of cyanmethemoglobin in optically clear solutions. The solutions are obtained by filtration of tissue extracts after the addition of ammonium sulfate to about half saturation.

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# The Occurrence and Estimation of Phytofluene in Plants

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Received September 11, 1945

## INTRODUCTION

The name "phytofluene" is proposed for a petroleum ether-soluble, colorless polyene with an isoprenic structure and a molecular weight of about 500. Its characteristic and sharp spectral maxima at 367, 348, and 331 m $\mu$  (in petroleum ether) were first observed in fractions of some petal extracts and a qualitative extinction curve has already been given (7). On chromatograms, phytofluene is adsorbed in about the same region as  $\alpha$ -carotene or some of its stereoisomers, and in ultraviolet light it displays a very intense, greenish-gray fluorescence on calcium hydroxide or alumina. A chemical characterization of this compound will be given elsewhere. However, in the present paper we intend to show that phytofluene is so widespread in the vegetable kingdom that, with some probability, it must be assigned a role in plant metabolism. Indeed, Table I, which includes a list of phytofluene-containing flowers, fruits and other organs, extends over nineteen plant families, and further investigation will doubtless increase this number.

Evidently, the occurrence and accumulation of phytofluene is not limited by systematic-botanical boundaries but (in part) by the following factors. So far we have been unable to detect phytofluene in chlorophyll-rich materials, such as, grass, spinach, green leaves or the needles of *Cedrus deodara* Lond. (A wild oat showed some traces.) The leaves of *Cinnamomum camphorum* Nees were tested in three stages of their development, *viz.*, young (still pinkish) leaves, then green and, finally, reddish autumnal leaves (before necrosis). None of these leaves of the same tree contained phytofluene.

TABLE I

*Examples for the Occurrence of Phytofluene in Plants*(The figures are based on  $E_{1\text{ cm.}}^{1\%} = 1200$ , at 348 m $\mu$ , in petroleum ether C. p. 60–70°)

Plant family	Plant	mg. Phytofluene in 1 kg. of fresh material
	<i>Petals</i>	
Bignoniaceae	<i>Bignonia</i> sp.	present
Bignoniaceae	<i>Tecomaria capensis</i> (Thbg.) Fenzl	present
Cannabaceae	<i>Canna</i> sp. (King Humbert var.)	0.2
Compositae	<i>Gazania rigens</i> R. Br.	32.5
Compositae	<i>Zinnia elegans</i> Jacq.	3.6
Loganiaceae	<i>Gelsemium sempervirens</i> Ait.	present
Papaveraceae	<i>Eschscholtzia californica</i> Cham. (California poppy)	5.0
Papilionatae	<i>Spartium junceum</i> L. (Scotch broom)	0.1
Scrophulariaceae	<i>Mimulus longiflorus</i> Grant (Monkey flower, pale yellow)	27.8
Spiraeoideae	<i>Photinia</i> sp.	0.5
Sterculiaceae	<i>Fremontia californica</i> Torr.	present
	<i>Fruits</i>	
Araceae	<i>Zantedeschia aethiopica</i> (L.) Spreng.	1.1
Cucurbitaceae	<i>Cucumis citrullus</i> L. (Watermelon, flesh)	2.2
Cucurbitaceae	<i>Cucumis Melo</i> L. (Cantaloup, flesh)	0.6
Cucurbitaceae	<i>Cucumis Melo</i> L. (Persian melon, flesh)	present
Cucurbitaceae	<i>Curcurbita maxima</i> Duch. (Squash, flesh)	present
Gramineae	<i>Zea mays</i> L. (Commercial yellow corn meal)	0.6
Myrtaceae	<i>Eugenia uniflora</i> Berg	0.7
Palmae	<i>Butia eriospatha</i> Becc. (without seeds)	0.3
Rosaceae	<i>Pyracantha yunnanensis</i> Schneid.	0.4
Rosaceae	<i>Pyracantha angustifolia</i> Schneid.	22.1, 23.0, 14.7, 27.7
Rosaceae	<i>Rosa canina</i> L. (without seeds)	1.8
Rosaceae	<i>Prunus domestica</i> L. (Plum, flesh)	1.0
Rosaceae	<i>Prunus persica</i> Sieb. Zucc. (Peach, flesh)	0.8
Rutaceae	<i>Citrus aurantium</i> Risso (Orange, juice)	0.3
Rutaceae	<i>Citrus aurantium</i> Risso (pigmented outer rind)	1.5
Rutaceae	<i>Citrus aurantium</i> Risso (white inner rind)	2.3
Solanaceae	<i>Capsicum annuum</i> L. (Red pepper, skin)	4.6
Solanaceae	<i>Capsicum annuum</i> L. (orange variety, skin)	present
Solanaceae	<i>Lycopersicum esculentum</i> Mill. (Tomato, unripe, without seeds)	2.0
Solanaceae	<i>Lycopersicum esculentum</i> Mill. (ripened at 19°; without seeds)	10.6
Solanaceae	<i>Lycopersicum esculentum</i> Mill. "San Marxano" used for canning (with seeds)	6.0
Solanaceae	<i>Lycopersicum esculentum</i> Mill. (Commercial canned tomato paste)	19.0, 28.5, 16.5, 14.5, 21.5, 18.4
	<i>Stems and roots</i>	
Convolvulaceae	<i>Cuscuta californica</i> Choisy (Dodder)	2.3
Umbelliferae	<i>Daucus carota</i> L. (Carrot)	7.3, 8.3

Likewise, negative results were obtained with some materials which contained neither chlorophyll nor carotenoids or only traces of the latter. To this group belong, for example, radishes, potatoes, apple flesh, whole wheat flour, white Marguerite petals, etc. Of course, it is possible that this delimitation will have to be shifted later.

So far as we know, phytofluene occurs chiefly in those plant organs which produce considerable amounts of carotenoid pigments in the absence of chlorophyll. Some petals contain as much as 25–32 mg. in 1 kilo of fresh material. For the purpose of isolation canned tomato paste which contains 15–30 mg. per kilo can be used. Much less phytofluene is present in carrot extracts; it certainly formed a component of a spectroscopically uncharacterized fluorescent oil obtained from carrots by Strain six years ago (5).

A chromatographic analysis of unripe and ripe *Pyracantha* berries revealed that the biosynthesis of phytofluene runs parallel with the

TABLE II  
*Polyene Content in Extracts of Unripe and Ripe Berries of  
*Pyracantha Angustifolia* Schneid.*

(Listed in the order of decreasing adsorption affinities on calcium hydroxide)

Polyene	mg. Polyene in 1 kg. of fresh berries	
	unripe	ripe
Lycopene	11.4	24.0
A poly- <i>cis</i> lycopene <sup>a</sup>	1.9	8.2
Unidentified <sup>b</sup>	3.7	9.0
Prolycopene	8.2	19.4
γ-Carotene	5.8	17.6
Neo-γ-carotenes	6.7	18.8
Unidentified <sup>c</sup>	0.9	—
Pro-γ-carotene	1.9	11.2
Unidentified <sup>d</sup>	0.9	2.6
Neo-β-carotene U	1.6	3.6
β-Carotene	15.0	16.0
Neo-β-carotenes	1.9	5.4
α-Carotene	present in small quantities	
Phytofluene	6.6	about 3–4
		14.7

<sup>a</sup> Visually observed spectral maxima in petroleum ether (b.p. 60–70°): 475, 446 mμ, and upon iodine catalysis, 501.5, 471.5, 443 mμ.

<sup>b</sup> 472.5, 444 mμ; with iodine, 471, 442.5 mμ.

<sup>c</sup> Only one band appeared, *viz.*, at 430 mμ.

<sup>d</sup> 458, 430 mμ; with iodine, blurred.

gradual formation of colored polyenes during the ripening process (Table II). The ratio of phytofluene content in ripe to that in unripe berries was found to be 2.2, while the corresponding ratio for the total carotenoid pigments was likewise 2.2; for lycopene it was 2.1, for prolycopene, 2.3, and for  $\gamma$ -carotene, 3.0;  $\beta$ -carotene and pro- $\gamma$ -carotene gave different figures. It seems that phytofluene is involved in the mechanism of the carotenoid formation but is not accumulated in the unripe fruit investigated.

Of course, plant extracts obtained with petroleum ether or similar solvents may contain fluorescing compounds of various types which can be differentiated by their relative adsorption affinities and spectra. Some petroleum ether-soluble fluorescing constituents of green leaves were detected by Strain (3, 4) who characterized them as follows: "Each fluorescing substance dissolved in cyclohexane exhibited strong absorption of ultraviolet light, particularly at shorter wave lengths but no maxima or minima were observed." This type is evidently different from the phytofluene which occurs, e.g., in carrots, and shows a sharp fine structure in the spectral region above 320 m $\mu$ . Similar substances appeared in some of our chromatograms and were mostly adsorbed in the top section of the Tswett column under our experimental conditions.

A third type of fluorescing compound has representatives in the orange or yellow petal of the common marigold (*Tagetes erecta* L.). These are characterized by their intensely sky blue or ink blue fluorescence, both in their solutions and adsorbates, and especially, by a single, rather broad spectral maximum which is located, for example, at 349 m $\mu$  or at 343 m $\mu$  (in hexane); no fine structure appeared in the extinction curves. The nature of such compounds will be studied. Those in *Tagetes* showed an adsorption affinity which was similar to that of phytofluene or somewhat weaker. In certain cases they seemed to be intermixed with minor amounts of phytofluene.

Only a few Cryptogams were tested so far by the methods outlined. No phytofluene was found in baker's yeast or white toadstool. Furthermore, the following animal products gave negative results: egg yolk, dried milk powder, pig's liver, commercial ox gall concentrate (from Brazil), sardine meal, sardine oil, dog fish oil, etc.

The suggested method for the determination of phytofluene in plant material is described below.

## EXPERIMENTAL

### *Estimation of Phytofluene in Plant Material*

Depending on the phytofluene and water content, the amount of fresh material needed for this purpose varies between 10 g. and 500 g. The smaller amount is satisfactory for some petals while the higher one is required when watermelon, prunes, corn meal, etc., are investigated.

The starting material was weighed after milling. In the presence of much water the extraction was preceded by a dehydration with 1-2 vol. of methanol. This was achieved by a 10 min. shaking or by keeping the material covered with methanol overnight. (Such treatment is unnecessary in the case of petals.) No phytofluene was lost in the methanol.

For the extraction, the material was shaken by hand in a bottle with equal parts of methanol and petroleum ether (b.p. 60-70°) (*cf.* 6); two liquid phases appeared when the material was not dry. Each of the three necessary extractions required 5 min. For example, for 10 g. of petals  $3 \times 50 + 50$  ml. of the solvents were used, and for 500 g. of watermelon flesh, after dehydration,  $3 \times 100 + 100$  ml. After each extraction the residue was pressed and washed with some methanol on a Büchner funnel. Larger amounts were handled in a basket centrifuge. About 1 vol. of water was cautiously added to the combined extracts until the pigment was transferred into the upper layer. (The aqueous fraction can be re-extracted with a little petroleum ether but this may be rendered difficult by emulsification.) The petroleum ether solution was dried with sodium sulfate and kept overnight in a broad Erlenmeyer flask over 20% methanolic KOH. Water was then cautiously added. The upper layer was washed alkali-free and concentrated *in vacuo* to 10-20 ml. while a slow stream of CO<sub>2</sub> was bubbling through (bath temperature, 40-45°). If a precipitate appeared (crystalline carotenoids, gummy substances, etc.), it was filtered off.

The colored solution was then poured onto a column (19 × 2.5 cm.) of a 4:1 mixture of calcium hydroxide (Shell Brand lime, chemical hydrate, 98% through 325 mesh) and alumina (Alorco, Grade F, minus 80 mesh). The chromatogram was developed with petroleum ether, in the course of  $\frac{1}{4}$ - $\frac{1}{2}$  hours, until an inspection with a portable ultraviolet lamp ("Wonderlite," 105-120 V, with light filter) showed that an intensely greenish-gray fluorescing bottom zone was well separated from the bulk of the carotenoid pigments. However, this zone usually was not free of  $\alpha$ -carotene or (and) some of its stereoisomers. The strongly fluorescing section was sometimes followed down the column by weaker ones which contained stereoisomeric phytofluenes and could not be neglected. After the column was cut with the aid of the ultraviolet lamp, the phytofluene was eluted with alcohol (*e.g.*, 100 ml.) on a fritted glass filter until the fluorescence of the flow had disappeared. Then 25 ml. of petroleum ether and water were added. The lower layer was checked in ultraviolet light and could be discarded in nearly all cases. The petroleum ether solution was washed free of alcohol in a continuous apparatus (2) or by repeated shakings; it was then dried and filtered into a volumetric flask.

In the Beckman photoelectric spectrophotometer (1) the compound should be identified by the position of its very sharp maxima at 348 m $\mu$

and 367-8 m $\mu$  and then estimated on the basis of  $E_{1\text{cm.}}^{1\%} = 1200$  at 348 m $\mu$  in petroleum ether (b. p. 60-70°). The possible presence of small amounts of carotenoids have little influence on such readings.

#### Acknowledgment

We wish to thank Mr. W. Hertrich, who placed the facilities of the Huntington Botanical Garden at our disposal, and Professor F. W. Went for tomato samples. We are also indebted to Mr. M. Gschwind and Mrs. Marydell Lipscomb for their assistance.

#### SUMMARY

The occurrence of a fluorescing polyene, "phytofluene," in vegetable tissue is discussed and a method for its estimation is described. Phytofluene is compared with other types of petroleum ether-soluble fluorescing constituents of plants which show characteristic features in their spectra.

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# **Granulocytopenia and Anemia in Riboflavin-Deficient Rats and Treatment with *L. Casei* Factor ("Folic Acid") and Riboflavin**

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Received September 12, 1945

## **INTRODUCTION**

It was found in previous studies (1) that granulocytopenia correctable by *L. casei* factor (L.C.F.) occurred only occasionally in rats fed highly purified diets. When sulfasuxidine or sulfaguanidine was included in such diets, the incidence of granulocytopenia was extremely high (1) (2). Anemia, also correctable by L.C.F., occurred in these rats in rather low incidence (2). When sulfadiazine or sulfathiazole was employed, the incidence of anemia was much higher (3). By the use of hemorrhage as a "load-test" in rats receiving sulfasuxidine, a severe inadequacy in red blood cell regeneration was uniformly demonstrable (4). In recent studies (5) on rats fed highly purified diets deficient in riboflavin, a similar, though less striking impairment in red blood cell regeneration was also found. The observation of anemia and granulocytopenia in a number of riboflavin-deficient control rats not subjected to hemorrhage together with our observations on the occurrence of anemia and granulocytopenia in pantothenic acid-deficient rats (6) prompted a further study of these blood dyscrasias.

Shukers and Day (7) found anemia in several riboflavin-deficient rats and also a decrease in lymphocytes. However, the lymphocytopenia was no greater in the riboflavin-deficient rats than in their riboflavin-supplemented, inanition controls. Jackson in his inanition studies (8) also noted a pronounced lymphocytopenia but no significant anemia or granulocytopenia. There have been a number of studies (9-14) on the influence of riboflavin on blood formation with conflicting results. As Shukers and Day point out, the results of these earlier studies are open

to question because diets were used which were possibly deficient in factors other than riboflavin.

In the present study of riboflavin-deficient rats, a severe granulocytopenia has been found which was correctable in almost all cases by crystalline L.C.F. but in only a few cases by riboflavin. A similar blood dyscrasia has also been found in riboflavin-supplemented, inanition controls. Severe anemia produced without recourse to hemorrhage has also been observed in riboflavin-deficient rats. It was corrected by riboflavin in some of the rats, while treatment with L.C.F. failed to correct the anemia in any of the rats tested.

#### EXPERIMENTAL

Weanling albino rats (Wistar or Osborne and Mendel) were placed on a riboflavin-deficient diet No. 963<sup>1</sup> or No. 990<sup>2</sup> as in previous studies (5). When weight loss, pallor or sluggish behavior suggested the possible presence of a blood dyscrasia, blood determinations were made as previously described (4).

For the purposes of this report, granulocytopenia was considered to be present when the polymorphonuclear granulocytes numbered 500 or less per cu. mm. Anemia was considered to be present when the hematocrit was less than 30 vol. per cent.

Of 185 rats fed the riboflavin-deficient diets, 18 died without a blood count having been made and are not considered further. Of the 167 rats studied, 54 were found to have granulocytopenia alone, 21 anemia alone, 26 granulocytopenia and anemia combined and 66 no blood dyscrasia. In an earlier study (1) of 187 rats fed highly purified diets containing riboflavin, 7 were found to have granulocytopenia alone and 1 granulocytopenia and anemia combined.

Treatment of rats with blood dyscrasias was by the daily, oral administration of 200 γ of riboflavin, of 25 γ or 100 γ of crystalline L.C.F.<sup>3</sup> (15) or of a combination of 200 γ of riboflavin and 25 γ of L.C.F. Treatment was begun on the day that the blood dyscrasia was noted and repeated daily for 3 days thereafter. Recounts were made on the day following the last treatment and in some experiments at intervals there-

<sup>1</sup> Diet No. 963 consisted of casein (Labco) 18.0%, Crisco 8.0%, salt mixture No. 550 (2) 4.0%, ferric citrate 1.16%, copper sulfate .5H<sub>2</sub>O 0.08% and sucrose 68.76%. Into 100 grams of this diet were incorporated 1 mg. of thiamin hydrochloride, 1 mg. of pyridoxine hydrochloride, 4 mg. of calcium pantothenate, 2 mg. of niacin, 200 mg. of choline chloride, 0.001 mg. of biotin and 0.4 mg. of 2-methyl-1,4-naphthoquinone. Twice weekly each rat received a supplement of 0.25 cc. of corn oil containing 2000 units of vitamin A and 200 units of vitamin D (Natola) and once weekly 3 mg. of α-tocopherol in 0.03 cc. of ethyl laurate.

<sup>2</sup> Diet No. 990 was the same as diet No. 963 except that anhydrous dextrose and leached, alcohol-extracted casein were used instead of sucrose and Labco casein respectively.

<sup>3</sup> A fermentation product furnished through the courtesy of Drs. E. L. R. Stokstad and B. L. Hutchings of Lederle Laboratories, Inc.

after. Among the treatment data are the results of treatment of rats which were in groups still under study. Data on incidence of dyscrasias in these groups are not included in this report.

*Granulocytopenia and Its Treatment* (Table I). Granulocytopenia, alone or combined with anemia, was noted in 80 of 167 riboflavin-deficient rats studied. The average time of onset was after 58 days (range: 21 to 128 days) of feeding the deficient diet. In granulocytopenic rats, there was usually a progressive decrease in weight with an increase in severity of the blood dyscrasia terminating in death.

TABLE I  
*Treatment of Granulocytopenia*

Treatment (daily for 4 days)	No. Rats Treated	No. Rats Dying During Treatment	Positive Re- sponses in Sur- vivors	Average Blood Counts Before and After Treatment			
				Total White Blood Cells per cu. mm.		Poly. Granulo- cytes per cu. mm.	
				Before	After	Before	After
None	22	10	8%	3330	2750	230	391
Riboflavin—200 γ	24	4	20%	3740	3378	250	440
Riboflavin—5 mg.	10	2	25%	4905	5506	245	500
L.C.F. ("Folic Acid")—25 γ	11	1	80%	2315	6910	205	3210
L.C.F. ("Folic Acid")—100 γ	26	3	96%	3585	10980	244	4070
Riboflavin—200 γ with L.C.F. ("Folic Acid")—25 γ	13*	1	83%	2430	9860	235	3110

\* Seven of these rats had failed to respond to treatment with riboflavin (200 γ) alone. All but one gave positive responses to the combined treatment.

A response to treatment of granulocytopenia was considered positive when a granulocyte level of 1000 cells per cu. mm. was attained 4 days after the start of treatment. Treatment with riboflavin alone resulted in few positive responses while treatment with crystalline L.C.F. was almost uniformly effective. Further recounts 4 to 8 days after the first recount of 9 rats which failed to respond to treatment with 200 γ of riboflavin revealed no increases in granulocyte levels. Seven rats which had failed to respond to treatment with riboflavin alone were treated with L.C.F. and riboflavin combined and 6 of these rats gave positive responses.

The presence of anemia or its development during the course of

treatment of a granulocytopenic rat did not appear to affect the granulocyte response.

During the 10 day period following the start of treatment, weight gains (5 to 56 grams) were noted in 13 of the 20 granulocytopenic rats which failed to respond to treatment with riboflavin. During the same period, weight losses (2 to 19 grams) were noted in 16 of the 30 granulocytopenic rats which responded favorably to treatment with L.C.F. The average weight gain in the entire riboflavin-treated group was 11.2 grams while the average weight loss in the entire L.C.F.-treated group was 1.7 gms.

*Relationship of Inanition to the Development of Granulocytopenia.* The debility and decline in weight noted in riboflavin-deficient, granulocytopenic rats suggested that inanition might be a factor in the development of this blood dyscrasia. Twenty-seven Osborne and Mendel male rats were all fed the riboflavin-deficient diet No. 990 and divided into 3 groups of 9, equal with respect to weight and litter distribution. Rats in group A were fed the diet without special supplement, rats in group B were supplemented with 200  $\gamma$  of riboflavin and pair-fed with litter mates in group A, and rats in group C were supplemented with 200  $\gamma$  of riboflavin and 20  $\gamma$  of L.C.F. and also pair-fed with litter mates in group A. Supplements were orally administered by pipette each day to each rat. Granulocytopenia was noted in 6 of the 9 riboflavin-deficient rats in group A. The same incidence was found among the riboflavin-supplemented, pair-fed rats in group B. Treatment of the granulocytopenia with L.C.F. resulted in a favorable response in every case. Granulocytopenia was not found among rats in group C receiving a preventive supplement of L.C.F.

*Anemia and Its Treatment* (Table II). Anemia alone or combined with granulocytopenia was noted in 47 of 167 riboflavin-deficient rats studied. The average time of onset was after 70 days (range: 30 to 140 days) of feeding the diet. Six anemic rats which were untreated, showed a progressive decline in hematocrit values and body weight and died within 12 days. Treatment was administered daily for 4 days and recounts were made at 4 and 10 days after the start of treatment.

A response to treatment of anemia was considered positive when an hematocrit level of 40 vol. per cent was attained 10 days after start of treatment. Of 22 rats treated with riboflavin, 5 died during the observation period, 10 gave positive responses and 7 failed to respond. In the latter group, recounts at 15 days after start of treatment of 5 rats

TABLE II  
*Treatment of Anemia*

Treatment (Daily for 4 days)	No. Rats Treated	No. Rats Dying*	Responses in Survivors	Blood Counts—Average and Range†					
				Hematocrit Vol. per cent			Hemoglobin G. per cent		
				0 Days	4 Days	10 Days	0 Days	10 Days	10 Days
Riboflavin—200 γ	22	5	Positive 10	23 <sup>10</sup> (14-29)	32 <sup>10</sup> (17-44)	43 <sup>10</sup> (40-49)	7.1 <sup>6</sup> (4.3-8.0)	13.5 <sup>6</sup> (10.8-15.8)	3.6 <sup>9</sup> (1.5-5.0)
			Negative 7	22 <sup>7</sup> (15-29)	19 <sup>7</sup> (13-28)	24 <sup>7</sup> (10-34)	6.3 <sup>5</sup> (3.3-9.0)	6.7 <sup>5</sup> (3.1-10.8)	3.1 <sup>4</sup> (1.5-5.3)
L.C.F. ("Folic Acid")— 25 γ or 100 γ ‡	11	4	Positive 0						
			Negative 7	31 <sup>7</sup> (10-45)	28 <sup>7</sup> (15-36)	23 <sup>7</sup> (11-30)			
Riboflavin—200 γ with L.C.F. ("Folic Acid") —25 γ or 100 γ §	8	2	Positive 4	20 <sup>4</sup> (11-27)	39 <sup>4</sup> (28-48)	46 <sup>4</sup> (40-50)			
			Negative 2	18.23 2	23.21	34.17			

\* Number dying during the 10 day observation period.

† The numerals above the average values indicate the number of animals which made up the average.

‡ Four rats were treated with 100 γ. Two of these rats died.

§ Four rats were treated with 100 γ. One rat died, one gave a positive response and two gave negative responses.

revealed 2 positive responses. Restriction of 12 of the 22 treated rats to a food intake of 4 grams daily, an amount approximating their intake prior to treatment, did not prevent favorable hematocrit responses in 7 of the 12 rats. (Granulocytopenia was noted in 2 of the rats which failed to respond and also in 2 rats which gave positive responses.) Treatment of 11 rats with L.C.F. resulted in no positive responses. Included in this group were 6 non-anemic, granulocytopenic rats which developed anemia (while their granulocyte dyscrasias were being corrected in each case). Five rats which remained anemic after treatment with L.C.F. were treated with riboflavin and L.C.F. combined and 4 of these rats gave positive responses.

Average values (with standard error) for mean corpuscular volume and mean corpuscular hemoglobin concentration of the anemic rats (Table II) were 61.2 cu.  $\mu$  (S.E. 2.55) and 31.8% (S.E. 0.88) respectively. Average values for mean corpuscular volume and mean corpuscular hemoglobin concentration of 13 rats fed a riboflavin-containing diet No. 956 (1) were 51.4 cu.  $\mu$  (S.E. 1.10) and 36.3% (S.E. 0.32) respectively.

#### DISCUSSION

By feeding a highly purified, riboflavin-deficient diet to rats, we have produced a deficiency state, characterized by granulocytopenia and correctable by L.C.F. We were also able to produce granulocytopenia in rather high incidence in riboflavin-supplemented rats pair-fed with their riboflavin-deficient litter mates indicating that diminished food intake is an important factor in the development of granulocytopenia. Further studies (16) have shown that the diminution of protein intake is a highly significant element in this inanition effect. With the available data, it remains difficult to evaluate the significance of the occasional positive granulocyte responses to treatment with riboflavin.

The correction of anemia in riboflavin-deficient rats by riboflavin indicates, in conjunction with previous data (5), the influence of riboflavin on red blood cell formation and regeneration. The mechanism whereby this influence is exerted remains obscure. The failure of some anemic rats to respond to riboflavin may possibly be indicative of an induced need for factors not contained in the diet.

It should be pointed out that the conclusions in this report are based upon the results of a treatment period of 4 days and an observation period of 4 to 10 days. The chief reason for such limited periods of

observation has been the poor survival of rats which fail to respond to treatment. It is possible, however, that longer and more vigorous treatment with more extended periods of observation might yield additional valuable information.

### SUMMARY

Granulocytopenia was noted in 80 of 167 rats fed a highly purified diet deficient in riboflavin. Crystalline *L. casei* factor ("folic acid") corrected this dyscrasia in 30 of 33 rats while riboflavin was effective in only 6 of 28 rats.

Granulocytopenia was also noted in high incidence among riboflavin-supplemented rats, pair-fed with their riboflavin-deficient litter mates. *L. casei* factor was effective in correcting this dyscrasia and in preventing it in other pair-fed, riboflavin-supplemented litter mates.

Anemia was noted in 47 of 167 rats fed a riboflavin-deficient diet. Riboflavin corrected the anemia in only 10 of 17 rats and was ineffective in the rest. Treatment with *L. casei* factor failed to correct the anemia in any of 7 rats tested.

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# The Inactivation of Iron by 2,2'-Bipyridine and Its Effect on Riboflavin Synthesis by *Clostridium acetobutylicum*

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Received October 1, 1945

## INTRODUCTION

Large-scale production of riboflavin by the fermentative action of strains of *Clostridium acetobutylicum* has been an industrial accomplishment for a number of years. Dried residues from butyl-acetonic fermentations have been prepared which contain up to about 6000 γ of riboflavin per gram (1) (2) (3).

The fact that the presence of small amounts of iron are extremely inhibitory to the extensive production of riboflavin by *Cl. acetobutylicum* has been observed by Arzberger (2), and subsequently by Rodgers (4). Arzberger's data on the fermentation of 5% cleaned corn mash shows that an addition of as little as 0.5 to 1 γ of iron per ml. as a salt was sufficient to reduce the riboflavin production from 25 to 28 γ/ml. to 8 to 12 γ/ml. With additions of 6 or more γ of iron per ml., the riboflavin produced amounted to about 1 to 2 γ/ml. He also observed that the addition of a cobalt salt inhibited riboflavin production in a manner similar to that of iron, while additions of salts of nickel, copper, lead and zinc to the extent of about 10 γ of the metal per ml. of medium had no significant effect on the production of riboflavin or the neutral volatile solvents. Large amounts of salts of these metals, however, did interfere with riboflavin production, but generally not until interference with the dissimilation of the carbohydrate was encountered.

Waring and Werkman (5) (6) (7) have recently studied the effect of iron deficiency on the aerobic and anaerobic metabolism of several organisms. Iron was removed from the basal medium by an 8-hydroxyquinoline-chloroform extraction method which they have described (5). They observed that the growth of *Aerobacter indo-*

*logenes* was a function of the iron concentration in the medium between 0 and 0.0225 p.p.m. Somewhat similar data were obtained for several other organisms.

Very recently Tanner, Vojnovich and Van Lanen (8), working with synthetic media from which iron was removed by the 8-hydroxyquinoline-chloroform method (5), described the very pronounced effect of exceedingly low concentrations of iron on riboflavin production by *Candida* species. Iron concentrations in the range of 0.005 to 0.01 γ per ml. resulted in the production of 120 to 216 γ of riboflavin per ml. of solution, while the organisms produced 15 or less γ/ml. when the iron concentration exceeded 0.1 γ/ml. This optimum iron level is about 100 times smaller than the optimum level for riboflavin production by *Cl. acetobutylicum*.

The exhaustive extraction of iron from a crude and heavy substrate such as corn mash, however, presents certain difficulties, particularly where it is undesirable to alter the medium in any other way.

2,2'-Bipyridine ( $\alpha,\alpha'$ -dipyridyl) is well known for its ability to form a very stable complex with ferrous iron. It has enjoyed rather widespread use as an analytical reagent for the determination of iron, but it has not been employed extensively other than analytically in biological studies involving iron.

## EXPERIMENTAL

### 1. Analytical Methods

The iron analyses of fermentation liquors were made by means of a modification of the method of Borgen and Elvehjem (9). It was desired to estimate only the available iron in solution.

A 10 ml. aliquot of the fermented liquor was treated with approximately 0.5 g. of trichloroacetic acid and after thorough agitation the mixture was filtered. Depending on the iron content of the filtrate, 1 to 3 ml. were combined with 1 ml. of a 0.0632% solution of 2,2'-bipyridine. To this mixture was added 2 ml. of a 20% sodium acetate-acetic acid buffer of pH 5. Distilled water was added to a final volume of 12 ml. Approximately 10 mg. of sodium dithionite ( $\text{Na}_2\text{S}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ ) were mixed with the sample immediately prior to the colorimetric determination. This reagent served the dual role of reducing essentially all of the available ferric iron to the ferrous state and of reducing riboflavin to the leuco form so that colorimetric measurements would not be complicated. The iron content was then estimated with an Evelyn photoelectric colorimeter, employing a 520 mμ. light filter. The values in γ of dissolved iron per ml. of medium were then taken from a standard curve.

The concentration of iron in the final dilution to be estimated was usually of the order of 0.1 to 0.5 γ/ml. At this dilution, dissociation of iron complexes other than that with 2,2'-bipyridine should be quite extensive so that the effects of interfering ions should be minimized. Sodium dithionite is a satisfactory reducing agent when the analysis is run with reasonable rapidity; otherwise colloidal sulfur may form and obscure the light transmission.

The analytical data for iron found in solution is approximate and is not intended to be highly critical in this particular study.

The riboflavin contents of the media were estimated fluorophotometrically on filtered and appropriately diluted but otherwise untreated liquor using the Coleman

fluorophotometer. It had been observed that trichloroacetic acid-treated, clarified media yielded low fluorophotometric riboflavin determinations.

The solvents, *n*-butanol, acetone and ethanol, were estimated as a mixture by distillation followed by a specific gravity measurement of the distillate.

### *2. Iron Effect on Riboflavin Production in Cereal Grain Mash*

Table I presents analytical data on iron found in the media as dissolved iron and its effect on riboflavin production by *Cl. acetobutylicum*.

It may be observed that the addition of 1 γ of iron per ml. is sufficient to reduce the riboflavin production from about 13 γ/ml. for the control to about 2 γ/ml., a drop of about 84%. Addition of 2 γ of

TABLE I

#### *Effect of Added Iron on Riboflavin Production by Cl. acetobutylicum*

Medium: Ground whole corn, 7 g./100 ml., steamed and sterilized.

Inoculum: 1% of a third generation culture of *Cl. acetobutylicum*, Strain 292.

Incubation: 44 hrs. at 37°C.

Flask	Added Fe γ/ml.	Iron Found γ/ml.	Riboflavin γ/ml.	Solids g./100 ml.	Solvents g./l.
1	0	1.1	13.8	1.93	15.0
2	1	1.5	2.5	2.16	14.5
3	2	1.5	0.5	2.28	13.5
4	4	4.2	0.6	2.04	15.0

iron per ml. reduces riboflavin production to about 0.5 γ/ml., which is near the lower production limit.

Discrepancies may be noted between the known amount of iron added in γ/ml. and the amount found by analysis of the filtrate. There is apparently, as might be expected, a distribution of iron between the solution and the suspended solids. It has been found that in media containing 2,2'-bipyridine along with the iron, some of the red complex is bound to some of the solids, particularly to the corn bran. Distinct reddish striations may be observed on the bran. Thus, where iron is added to fermentation media and analysis is made on clarified liquor, that which is found does not represent total iron, but total iron found in solution.

### *3. Antagonism of Iron and 2,2'-Bipyridine in Grain Mash Fermentations*

A number of reagents were studied to determine their ability to inactivate iron as indicated by increased riboflavin production in the

medium by *Cl. acetobutylicum*. Among agents studied and found to be unsuccessful were cupferron, 7-iodo-8-hydroxyquinoline-5-sulfonic acid, and the anions, cyanide, oxalate, thiocyanate, sulfide and citrate. The employment of 2,2'-bipyridine (Eastman Kodak Company) was highly successful. The inhibitory effects of excessive iron on riboflavin production by *Cl. acetobutylicum* are given in Table II, and the ability of 2,2'-bipyridine to inactivate the iron and restore the riboflavin-producing ability of the biological system is clearly shown.

It may be observed from the data of Table II that as the iron is progressively inactivated by 2,2'-bipyridine, the biosynthesis of riboflavin increases where fermentation is complete as evidenced by reasonable constancy in the production of solvents and in the solids content of the fermented liquor. When the metabolism is thus suppressed, presumably because of insufficient available iron, the riboflavin production also decreased. Decreasing the available iron by the action of increasing amounts of 2,2'-bipyridine gradually slows the fermentative rate and ultimately inhibits metabolic action if sufficient 2,2'-bipyridine is employed.

This remarkable increase in riboflavin production in low iron-containing media may be indicative of two alternative dissimilation mechanisms not necessarily entirely independent of each other, one involving an ionizable ferrous structure, and the other involving a riboflavin structure, probably a flavoprotein. The highly anaerobic nature of this organism should eliminate any sort of a cytochrome system.

That this relationship is not unique for a single strain of *Cl. acetobutylicum* is indicated in Fig. 1. The organism employed here is strain 3 which has a higher riboflavin producing ability than strain 292.

No exceptions have been found as yet to this action of iron on riboflavin-producing abilities of the cereal grain-fermenting strains of *Cl. acetobutylicum*, although it has been possible to obtain strains which are outstandingly better riboflavin producers than others.

Saunders and McClung (10), in support of the work of Rodgers (4), have reported that under their conditions the addition of small amounts of iron to corn mash stimulated riboflavin production by four out of five strains of *Cl. acetobutylicum*. This was not true, however, for *Cl. roseum*, *Cl. felsinum*, and certain other anaerobes.

From the data of Table II it may be observed that about 9.5 γ of 2,2'-bipyridine are required to inactivate 1 γ of ferrous ion. Stoichio-

TABLE II

*2,2'-Bipyridine Inhibition of Iron Antagonism to Riboflavin Biosynthesis*

Medium: Ground whole corn, 7 g./100 ml., 200 ml. per 300-ml. flask. Steamed for 1 hr. and sterilized for 1 hr. at 15 lb./in<sup>2</sup>. Corn analysis: 12.7% H<sub>2</sub>O and 72.4% starch (dry basis).

Inoculum: 2.5% of a fourth generation culture of *Cl. acetobutylicum*, Strain 292, developed on the above medium.

Incubation: 44 hrs. at 37°C.

Added Fe γ/ml.	Added 2,2'-Bipyridine γ/ml.	Iron Found in Solution γ/ml.	Solvents γ/l.	Riboflavin Produced γ/ml.	Total Solids g./100 ml.
0	0	1.1	15.0	13.8	1.93
	0	1.1	14.5	16.0	2.41
	3.2	0.9	14.5	11.5	2.62
	6.3	0.8	13.5	15.0	2.28
	12.6*	0.9	10.0	14.8	3.32
	19.0	0.9	6.5	11.3	4.26
	25.3	0.7	0.5	3.5	6.00
	31.6	0.95	0	1.3	6.36
	1	1.5	14.5	2.5	2.16
	3.2	1.5	15.0	2.1	2.16
1	6.3	1.2	15.0	5.5	2.24
	12.6	1.4	14.5	10.6	2.04
	19.0	1.1	13.5	15.0	2.16
	22.1*	1.1	11.0	16.0	3.28
	25.3	1.3	8.5	12.8	3.80
	28.4	1.0	4.0	7.5	4.80
	31.6	0.8	0.5	3.3	5.76
	37.9	0.8	0	1.9	6.40
	2	1.5	13.5	0.5	2.28
	6.3	1.5	15.5	0.8	2.28
2	12.6	1.8	15.5	2.0	2.28
	19.0	1.7	15.0	5.3	2.00
	25.3	1.4	14.5	13.4	2.04
	28.4	2.0	13.5	12.5	2.16
	31.6*	1.9	10.0	12.3	3.40
	34.8	2.3	9.5	14.5	3.36
	37.9	3.3	8.0	13.9	3.36
	44.2	2.3	2.0	3.0	5.92
	4	4.2	15.0	0.6	2.04
	12.6	4.1	15.5	0.5	2.04
4	19.0	3.9	15.5	0.6	2.08
	25.3	4.2	16.5	0.65	2.20
	31.6	3.9	16.5	0.6	2.04
	37.9	3.8	15.5	2.5	2.00
	44.2	3.3	15.5	11.6	2.32
	50.6*	3.5	13.0	14.2	2.88
	56.9	3.0	5.0	5.4	4.28
	63.2	3.4	1.5	2.4	5.92

\* Lowest level of 2,2'-bipyridine at which significant suppression of fermentation occurred. (See Table IV.)

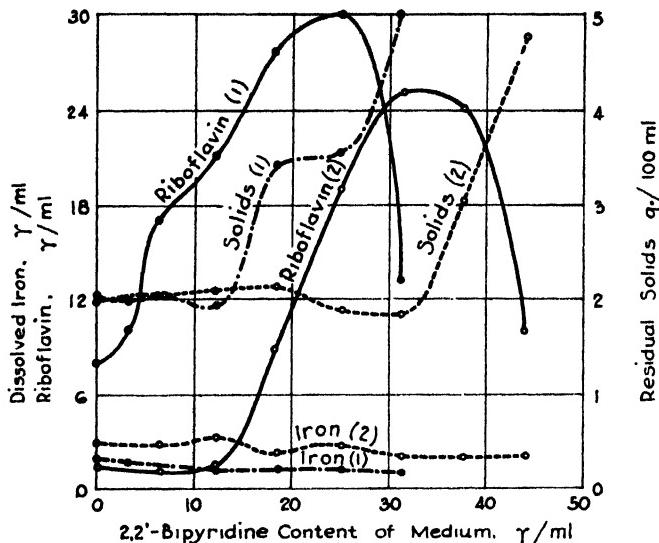


FIG. 1

## 2,2'-Bipyridine Inhibition of Iron Antagonism to Riboflavin Biosynthesis

Medium: 7% Ground Whole Corn.

Inoculum: 2% of Fourth Generation of *Cl. Acetobutylicum*, Strain 3.

Incubation: 44 Hrs. at 37°C.

Curves 1: No Iron Added.

Curves 2: 2 γ of Iron Added per ml.

metrically, 8.4 γ should be required, but an excess of 2,2'-bipyridine should suppress dissociation of the ferrous-dipyridyl complex according to the mass law. The data are presented in Table III.

TABLE III

*Requirement of 2,2'-Bipyridine to Biologically Inactivate Added Iron*

The media listed are those of Table II (marked by an asterisk) to which just sufficient 2,2'-bipyridine has been added to noticeably interfere with solvent production and to significantly increase the residual solids.

Added Iron γ/ml.	Added 2,2'-Bipyridine γ/ml.	2,2'-Bipyridine Required to Inactivate Added Iron		
		γ/ml.	γ/γ	moles/mole
0	12.6	—	—	—
1	22.1	9.5	9.5	3.4
2	31.6	19.0	9.5	3.4
4	50.6	38.0	9.5	3.4

#### 4. Iron Effect on Riboflavin Production in Molasses Media

That the aforementioned data are not typical of all butylacetonic processes is indicated in the fermentation of molasses media. The cultures employed here are selected molasses-fermenting strains of *Cl. acetobutylicum*, and are designated in patent literature as *Clostridium saccharo-butyl-acetonicum-liquefaciens* (11) and *Clostridium saccharo-butyl-acetonicum-liquefaciens-delta* (12).

TABLE IV  
*The Antagonism Between Iron and 2,2'-Bipyridine During Production of Riboflavin in the Molasses Fermentation*

Molasses: Cuban High Test, 75.3% sugar as sucrose.  
 Medium A: 7.17% Molasses (5.4% sugar), 0.3%  $(\text{NH}_4)_2\text{SO}_4$ , 0.05%  $\text{K}_2\text{HPO}_4$ , and 0.4%  $\text{CaCO}_3$  in tap water.  
 Medium B: 8.64% Molasses (6.5% sugar), plus salts and water as in Medium A.  
 Preparation: 100 ml. of medium in 125 ml. flasks.  
 Inoculum: 2% of a fifth generation culture as indicated.  
 Incubation: 72 hrs. at 32°C.

Iron Added $\gamma/\text{ml.}$	2,2'-Bipyridine Added $\gamma/\text{ml.}$	Iron Found $\gamma/\text{ml.}$	Riboflavin Found $\gamma/\text{ml.}$	Final Solids $\text{g./100 ml.}$
<i>Clostridium saccharo-butyl-acetonicum-liquefaciens-delta</i> (12) on Medium A				
0	0	--	2.05	2.24
	3.2	--	1.75	2.32
	6.3	--	1.85	2.28
	12.6	1.6	1.6	2.28
	31.6	0.8	0	5.12
1	0	1.45	1.55	2.28
	3.2	2.6	1.75	2.36
	6.3	--	1.50	2.28
	12.6	--	1.30	2.28
	25.2	--	1.0	2.20
	44.2	0.44	0.25	5.28
<i>Clostridium saccharo-butyl-acetonicum-liquefaciens</i> (11) on Medium B				
0	0	--	1.5	2.80
	3.2	2.13	1.5	2.88
	6.3	--	1.25	2.68
	12.6	2.0	1.75	2.48
	31.6	0.6	1.5	5.96
1	0	--	1.5	2.84
	3.2	--	1.25	2.48
	6.3	2.7	1.75	2.48
	12.6	2.9	1.5	2.36
	31.6	--	1.75	2.64

Data in Table IV show that sufficient 2,2'-bipyridine had been included in certain of the media to suppress or inhibit dissimilation of the carbohydrate as evidenced by the high final solids. A low amount or cessation of gas evolution was also observed during the incubation. However, in no instance was there even the slightest indication of any improvement in riboflavin production by these clostridia. The reason for the absence of increased riboflavin production upon inactivation of the iron is uncertain. It may be characteristic of the organism, or be caused by excessive amounts of another inhibitor such as cobalt, or it may be caused by the absence of certain essential agents in the molasses media which are found in grain.

### DISCUSSION

It has been demonstrated that ionic ferrous iron can be selectively inactivated in certain biological media by the employment of 2,2'-bipyridine as a complex forming reagent. It is possible that this reagent may be of value for the selective inactivation of ferrous iron in other biological studies. The great stability and low volatility of the reagent make it quite convenient for use in microbiological media which requires sterilization.

A degree of uncertainty still exists in assigning all inhibitory action of 2,2'-bipyridine exclusively to the complex forming action with ferrous iron. Such ammines of other cations are known to exist. The inhibitory action of 2,2'-bipyridine can be overcome by the addition of more iron, however.

If the cessation of fermentation by *Cl. acetobutylicum* is brought about by the action of 2,2'-bipyridine on dissociable ferrous iron exclusively, then certain conclusions might be drawn:

- (1) Iron is essential for metabolism of *Cl. acetobutylicum*.
- (2) The appearance of increasing amounts of riboflavin coincident with a decrease in the available iron of the system may be indicative of alternate hydrogen carrier systems, one of which would involve a dissociable ferrous complex which must exhibit a higher degree of dissociation than the ferrous-bipyridine complex. As long as the iron-containing system is available to the organism it maintains a minimal riboflavin concentration; but when the iron system becomes decreasingly available, the organism may attempt to survive by expansion of its riboflavin or flavo-protein system.

Of the clostridia studied (13), they, being anaerobes, are characterized in part by the absence of many enzyme systems concerned with the utilization of molecular oxygen, particularly catalase, peroxidase, cytochrome and cytochrome oxidase.

Waring and Werkman (7) in their studies on iron deficient *Aerobacter indologenes* have found no evidence of any of the cytochrome system, and the catalase and peroxidase activities were less than 5% of their normal values. They also observed a deficiency of the anaerobic formate splitting system.

### SUMMARY

2,2'-Bipyridine is capable of inactivating iron in the fermentation of corn mash by *Cl. acetobutylicum* so that a marked increase in riboflavin production is promoted.

Although 2,2'-bipyridine inactivates iron in the molasses fermentation by industrial molasses-fermenting strains of *Cl. acetobutylicum*, an increase in riboflavin production has not been observed.

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# Infection by Viruses

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Received October 2, 1945

## INTRODUCTION

One of the questions frequently asked about viruses is how they multiply, an issue concerning which there is meager experimental evidence to afford a basis for speculation. Insight into its fundamental aspects may be gained from an answer to the simpler question of whether infection is induced by the interaction of the host protoplasm with a single virus particle or with many particles. While there is disagreement in the literature about this matter, considerable experimental evidence can fortunately be brought to bear upon it.

## THEORETICAL

*Poisson Series and Infectivity:* Let it be assumed that the occurrence or non-occurrence of a virus infection is due solely to the chance presence or absence of at least the minimum requisite number of infectious units in a particular element of volume favorably situated with respect to the host. The theoretical problem then involves the calculation of the probability that at least one, two, or some other number of virus particles will occur within a particular volume element. This problem has been considered in relation to bacterial infections by Greenwood and Yule (1) and Halvorson and Ziegler (2). Their treatment serves as a model for deriving the equations applicable to the study of viruses.

Consider a solution containing  $n$  virus particles per unit volume diluted to a relative concentration of  $x$  and a volume of  $V$ . Let the volume of one virus unit be

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$a$  and the bulk of the solution be divided into volume elements of size  $a$ . The probability that one virus unit will be found in a particular volume element chosen at random is the total number of virus particles divided by the total number of volume elements,  $\frac{Vnx}{V} = \frac{nx}{a}$ . The probability of not finding a virus particle will be  $1 - anx$ .

If a volume of  $v$ , that is  $\frac{v}{a}$  volume elements, is examined, the probabilities of finding 0, 1, 2 ...  $n$  virus particles will be given by the successive terms of the expansion of the binomial,  $[(1 - anx) + anx]^{\frac{v}{a}}$ . The first term,  $(1 - anx)^{\frac{v}{a}}$  is the probability of finding no virus particles in a volume of  $v$ , the second,  $\frac{v}{a} anx (1 - anx)^{\frac{v}{a}-1}$  is that of finding one, and the third,

$$\frac{\frac{v}{a} \left( \frac{v}{a} - 1 \right) (anx)^2 (1 - anx)^{\frac{v}{a}-2}}{2!}$$

is that of finding two.

Consider the probability,  $P_0$ , of getting no particles.  $P_0 = (1 - anx)^{\frac{v}{a}}$ , and  $\log_e P_0 = \frac{v}{a} \log_e (1 - anx)$ . Since  $\log_e (1 + x) = x - \frac{x^2}{2} + \frac{x^3}{3} \dots$ ,

$$\log_e P_0 = \frac{v}{a} \left( -anx - \frac{(anx)^2}{2} - \frac{(anx)^3}{3} - \dots \right).$$

When  $anx$  is much less than unity, the higher order terms become negligible and then  $\log_e P_0 = -nvx$ , or  $P_0 = e^{-nvx}$ . The probability of there being at least one virus particle in  $v$  is, therefore,  $1 - e^{-nvx}$ .

The second term of the expanded binomial is the probability,  $P_1$ , that there will be exactly one particle in  $v$ .  $P_1 = nvx(1 - anx)^{\frac{v}{a}-1}$ . When it is transformed to logarithms and expanded as above, it becomes

$$\log_e \left( \frac{P_1}{nvx} \right) = \left( \frac{v}{a} - 1 \right) \log_e (1 - anx) = \left( \frac{v}{a} - 1 \right) \left( -anx - \frac{(anx)^2}{2} - \frac{(anx)^3}{3} \dots \right).$$

When  $\frac{v}{a} - 1$  is about equal to  $\frac{v}{a}$  and  $anx$  is small,  $\log_e \left( \frac{P_1}{nvx} \right) = -nvx$ . Or  $P_1 = nvxe^{-nvx}$ . The probability that there will be either zero or one particle in  $v$  is  $e^{-nvx} + nvxe^{-nvx}$ . Thus, the probability that there will be two or more is  $1 - e^{-nvx} - nvxe^{-nvx}$ . It is evident that the terms  $P_0$ ,  $P_1$ ,  $P_2$ , etc., are the terms of the Poisson series.

*Local Lesions as Criteria:* Broadly speaking, there are two distinct types of situation to which these equations may be applied. The simplest is that in which  $N$  inoculations, each of volume  $v$ , are made into  $N$  susceptible regions in a way that every virus particle will be afforded an opportunity to infect. If  $y$  of the  $N$  inoculated regions develop infection,  $\frac{y}{N}$  will be an experimental estimate of the proba-

bility of each volume causing an infection. If infection is due solely to the chance occurrence of the requisite number of particles,  $\frac{y}{N}$  can be equated to one of the *a priori* probability expressions derived from the Poisson series to give, for one particle,  $\frac{y}{N} = 1 - e^{-nvx}$  (Equation 1), and for two particles,  $\frac{y}{N} = 1 - e^{-nvx} - nvxe^{-nvx}$  (Equation 2). It may be observed in this case that  $y$ ,  $N$ ,  $x$ , and  $v$  are all determined experimentally. It is therefore possible, if the assumptions are granted, not only to decide which of the equations fits the experimental data, but also to determine the value of  $n$ . Thus, the technique affords an absolute method of measuring the concentration of infectious units. It was used in this form by Parker (3) in studies on vaccinia virus.

The more complex type of situation to which these theoretical considerations may be applied is that which is encountered when an undetermined amount of the virus suspension at a relative concentration of  $x$  is spread over a surface containing  $N$  susceptible regions. In this special case one must be concerned with the probability that the requisite number of virus particles will be found in elements of volume of unknown but constant value,  $v$ , immediately adjacent to each of the susceptible regions. These elements of volume have no relationship whatever to the total volume of virus solution applied to the total surface. If a series of inoculations is made at several relative concentrations  $x$ , the number of local responses,  $y$ , can be counted for each concentration and considered as a function of  $x$ . In attempting to fit data of this sort to either Equation 1 or 2, it is necessary to assign the proper values to the constants  $N$  and  $(vn)$ . The constant  $N$  can be determined experimentally by applying higher and higher concentrations of virus to successive surfaces until the number of local responses per surface no longer increases with increasing concentration. Both  $N$  and  $vn$  can be evaluated from the data of the dilution curves themselves, for it is a common practice to assign definite values to at least two arbitrary constants in fitting data to mathematical equations. However, the value obtained for  $N$  by either method will be an average, since  $N$  may vary considerably. Only by using a large number of surfaces at each dilution can the average value of  $N$  be regarded as a reasonable basis for an experimental estimate of the probability of infection at a particular dilution. This fact is very

significant, for it introduces an unavoidable experimental error into the evaluation of  $\frac{y}{N}$ . Nevertheless, it is theoretically possible to determine which of the probability equations, if any, fit the data and thus to decide the minimum number of particles required for infection. It is not possible to determine  $n$ , because the value of  $v$  is not known. As examples of this more complex type of situation, Youden, Beale, and Guthrie (4) and later Bald (5) applied Equation 1 to the dilution data of numerous plant viruses.

*Generalized Infection as a Criterion:* A slight modification of the situation discussed above is that in which the surface containing the  $N$  susceptible regions is itself regarded as the susceptible region. As has been shown previously, if a virus solution is applied over a surface containing  $N$  susceptible regions, the probability that the  $i$ th susceptible region will escape infection (if one particle is sufficient to induce an infection) is  $e^{-vnz}$ . The probability that all  $N$  regions on that particular surface will escape infection is obviously  $e^{-Nvnz}$ . Therefore, the probability that the surface as a whole will become infected is  $1 - e^{-Nvnz}$ . If  $M$  such surfaces are inoculated and  $L$  become infected,  $\frac{L}{M}$  will be the experimental estimate of the average probability of a surface becoming infected. The *a priori* probability, on the other hand, will be  $\frac{1}{M} \sum_0^M [1 - e^{-Nvnz}]$ . Only if  $N$  is the same for every surface will this *a priori* probability reduce to  $1 - e^{-Nvnz}$ . When equated to  $\frac{L}{M}$ , an expression of the same form as Equation 1 is obtained.

Bald (5) applied Equation 1 to data on various plant viruses in which leaves or half leaves were regarded as susceptible units, and Bryan and Beard (6) attempted to apply it to data on rabbit papilloma virus in which areas of scarified skin were regarded as the susceptible units. Neither pointed out the exact nature of the assumption involved.

*Aggregation:* When the *a priori* probabilities of infection are plotted against the logarithm of dilution according to Equations 1 and 2 and higher members of the series, a group of sigmoidal curves, which differ from one another principally with respect to the slope of the central portion, is obtained for the cases of 1, 2, 3, etc., particles being the minimum requirement for infection (Fig. 1). Experimental infectivity dilution curves usually have this same general shape when

plotted in the above manner. Indeed, many of the experimental data at present available can be fitted to the graph of Equation 1. There are, however, dilution data for plant viruses that do not follow the course of Equation 1 exactly. Bald (7) concluded that this distortion of the dilution curve might be explained on the assumption that some virus samples are partially aggregated, the degree of aggregation varying with the sample, and that the aggregates dissociate into active

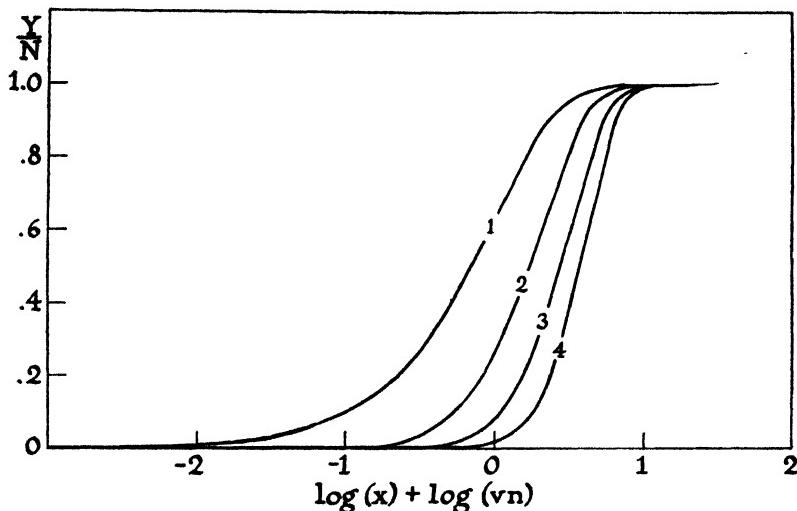


FIG. 1

Graphs Showing the Probability, Plotted as a Function of  $\log_{10}$  of Concentration, of Finding within a Volume  $v$  a Minimum of 1 (Curve 1), 2 (Curve 2), 3 (Curve 3), or 4 (Curve 4) Particles  
Curves 1 and 2 are graphs of Equations 1 and 2, respectively.

particles when the sample is diluted. An equation similar to Equation 1 was derived to cover these particular cases.

Let  $y$ ,  $N$ ,  $v$ ,  $n$ , and  $x$  have the same meaning as in Equation 1. Designate the number of aggregates per unit volume containing *one or more than one* constituent virus particle by the symbol  $b$ . Then, if it is assumed that each aggregate behaves biologically like a single unit regardless of the number of constituents it contains,  $bx$  will be the number of biologically active aggregates and  $nx$  the total number of particles in a unit volume of a solution of relative concentration  $x$ . Therefore Equation 1 may be rewritten,  $\frac{y}{N} = 1 - e^{-bx}$ .

To solve  $vbx$  in terms of  $vnx$ , one must consider the number of bonds,  $r$ , in unit volume binding virus particles into aggregates. It is clear that  $r = nx - bx$ , for if all the virus units were bound into a single aggregate,  $nx - 1$  bonds would be required, etc. If the breaking or closing of a bond is independent of the chain length, one can write,  $\frac{dr}{dt} = k_1(bx)^2 - k_2r$ . At equilibrium,  $\frac{dr}{dt} = 0$ , hence,  $r = \frac{k_1}{k_2}(bx)^2$ . Since  $r = nx - bx$ ,  $\frac{k_1}{k_2}(bx)^2 + bx - nx = 0$ . Multiplying by  $v$ , substituting  $K$  for  $\frac{k_1}{k_2}v$ , and solving for  $vbx$ , there is obtained  $vbx = \frac{-1 \pm \sqrt{1 + 4Kvnz}}{2K}$ . Only positive values of  $vbx$  have any physical meaning. Hence, in place of Equation 1 we may write  $\frac{y}{N} = 1 - e^{-(\sqrt{1+4Kvnz}-1)/2K}$  (Equation 3). Bald has fitted, within the limits of experimental error, data for dilution experiments with two different strains of tobacco mosaic virus to Equation 3. The values of  $K$  obtained varied between 0.25 and 17.

*Gaddum Theory Applied to Infectivity:* The question arises as to what extent, in the favorable cases, the agreement of experimental results to Equation 1 justifies the conclusion that the mechanism postulated in the derivation is really operative. This question is of particular importance because an alternative theory has been proposed which can account for the sigmoidal shape of the infectivity curves. Bryan and Beard (6) have pointed out that the theory developed by Gaddum (8) to explain the sigmoidal shape of the graph relating the response of organisms to drug doses can be adapted to the case of the response of hosts to virus doses. According to this view, a great number of virus particles come into contact with a susceptible region in a host, and the host brings into play defense mechanisms to combat the invaders. It is postulated that the susceptibility of the host varies from individual to individual, and that the distribution of the logarithms of dose susceptibilities follows the normal law. Suppose that the dose of virus which causes 50% infection is called unity and that the standard deviation of the distribution of susceptibilities is 0.5 logarithms. From the properties of the normal curve it can be shown that when series of hosts are inoculated with doses of 1/100, 1/10, 1/3, 1, 3, 10, and 100 times the unit dose, 0, 2, 17, 50, 83, 98, and 100%, respectively, should succumb. If the above percentages are plotted against logarithm of dose, a sigmoidal curve will be obtained. Furthermore, for the special case chosen here, this curve will be similar in general shape to the graph of Equation 1, as is shown in Fig. 2. If a different standard deviation had been chosen, a sigmoidal curve of different slope would have been obtained.

## AGREEMENT OF THEORIES WITH OBSERVED DATA

*Local Infections:* To test most adequately the applicability of Equations 1 and 2, it is desirable to choose a virus-host system which provides unambiguous symptoms of infection and for which the method of inoculation allows a definite volume of virus suspension to be introduced into a susceptible tissue in a manner that provides every virus particle an opportunity to infect. The closest approach to this ideal,

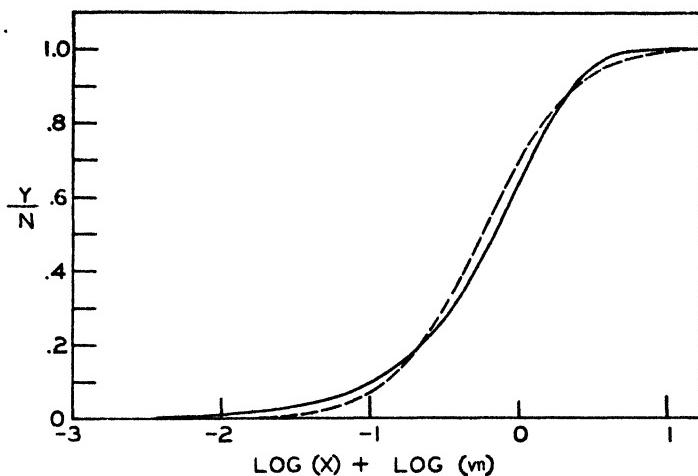


FIG. 2

Graphs Showing the Probability of Virus Infection Plotted as a Function of  $\log_{10}$  of Virus Concentration

Solid line is identical with curve 1 of Fig. 1. Broken line corresponds to the expectation based on the theory that dose response is due to susceptibility variation of the host, for the case in which  $\log$  of susceptibility is distributed normally with a standard deviation of 0.5.

of which the authors have knowledge, is afforded by Parker's (3) study of the Board of Health strain of vaccinia virus, a strain highly virulent for rabbits inoculated intradermally. In this case 0.25 cc. portions of virus suspension at various concentrations were injected intradermally into rabbits. Severe necrotic lesions were formed. The results are presented graphically in Fig. 3. The smooth curve fitting the data is a graph of Equation 1. Every quantity in the equation except  $n$  can be evaluated experimentally. Haldane (9) developed an

algebraic method for calculating  $n$  and obtained a value of  $76 \times 10^6$  infectious units per cc. with a standard error of  $6 \times 10^6$ .

The goodness of fit of Parker's data to the graph of Equation 1 can be evaluated objectively by using the  $\chi^2$  criterion. Haldane did this and found a value of 3.53 for  $\chi^2$  for 6 degrees of freedom. This corresponds to a probability of 0.74 that the deviations observed are due entirely to chance. It affords an objective confirmation of the com-

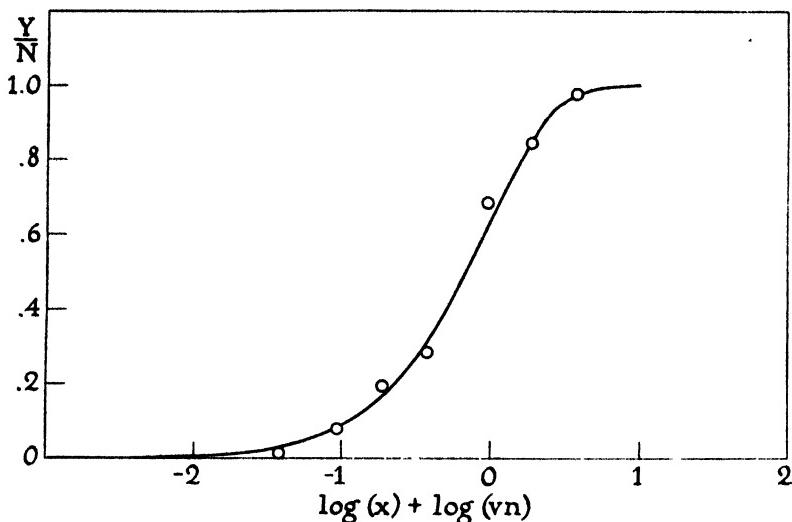


FIG. 3

Fraction of Vaccinia Virus Inoculations that Developed Lesions, Plotted as a Function of  $\log_{10}$  of Virus Concentration  
Parker's data for Board of Health Strain. Curve is identical with curve 1 of Fig. 1.

pability of Parker's data on the Board of Health strain of vaccinia virus with the theory that only one virus particle is necessary to cause an infection. Haldane has calculated  $\chi^2$  for fit of the data to the graph of Equation 2. The value obtained is so high that the data are incompatible with it even by the most conservative standards.

Although Parker's data obtained with the Board of Health strain are in excellent agreement with the assumption that one vaccinia virus particle is sufficient to initiate infection, they do not in any sense prove that every virus particle introduced intradermally will

infect. The results make it necessary to assume only that the probability that a virus particle will find a favorable environment is essentially a constant from site to site. Smadel, Rivers and Pickels (10) studied the ratio of the number of infectious units to elementary bodies for seven preparations of the *CL* strain of vaccinia virus. This strain is also extremely virulent for rabbits inoculated intradermally.

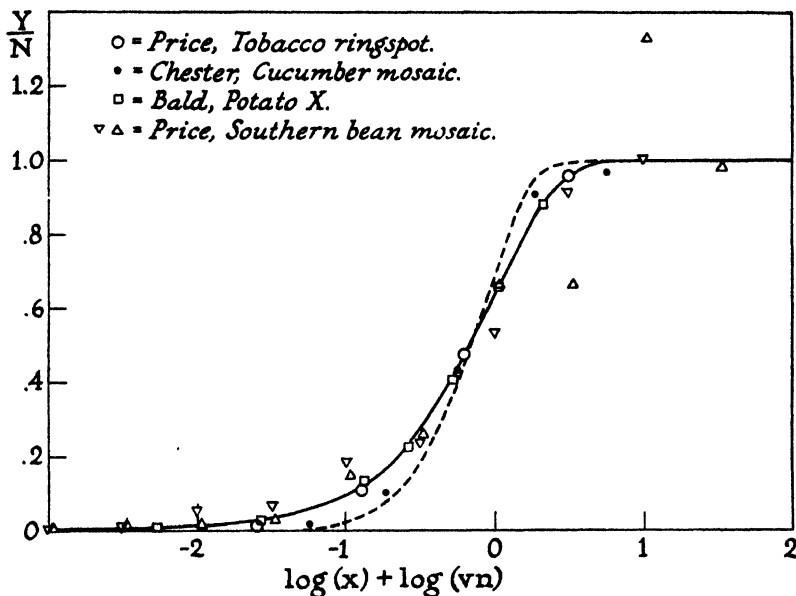


Fig. 4

Experimental Probability of Infection with Various Plant Virus-Host Systems  
Plotted as a Function of  $\log_{10}$  of Virus Concentration

Solid line is identical with curve 1 of Fig. 1 and broken line is identical with curve 2 of Fig. 1.

The ratios obtained varied from 1/2.4 to 1/9.7, with an average of 1/4.2. If it is assumed that the elementary body is the infectious unit, this result means either that the extent of aggregation plus inactivation of the elementary bodies is less than 75% or that the probability that an infectious unit of *CL* virus introduced intradermally will find an opportunity to infect is not less than 0.25. These results can not possibly be interpreted to mean that four or more infectious units

must be present for a lesion to develop, for were that the case, the dilution curve would have to be very much steeper than it is.

In testing the goodness of fit of Equation 1 to infectivity data for plant viruses, we are confronted with a situation somewhat different from that just discussed, for the virus suspension is not injected but is rubbed across the leaf surface. The rubbing spreads a film of an unknown amount of virus solution and causes numerous minute

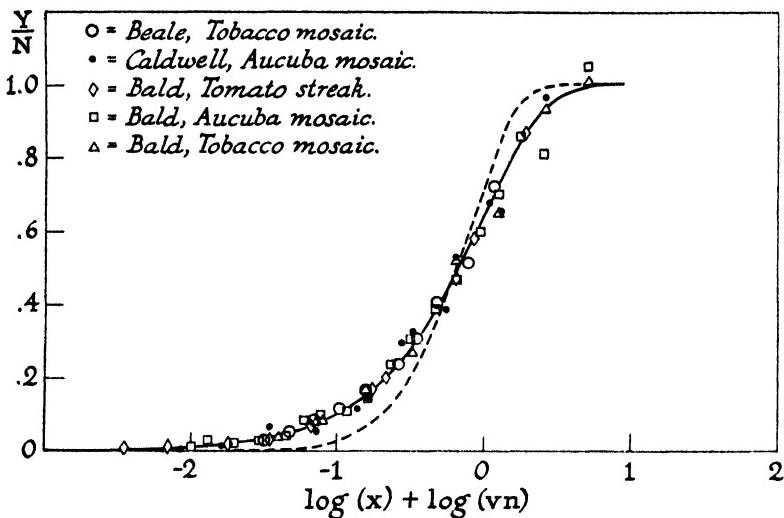


FIG. 5

Experimental Probability of Infection with Various Plant Virus-Host Systems  
Plotted as a Function of  $\log_{10}$  of Virus Concentration

Solid line is identical with curve 1 of Fig. 1 and broken line is identical with curve 2 of Fig. 1.

injuries. Experimental evidence indicates that, for a specific region to become infected, it must be injured to a sufficient but not to too great an extent (11, 12, 13).

Infectivity data for various strains of tobacco mosaic virus on *Nicotiana glutinosa* L. were selected from the literature (4, 14) and are presented in Fig. 4. Comparable data for ringspot virus on *Vigna sinensis* (L.) Endl., cucumber mosaic virus on *V. sinensis*, potato X virus on *N. glutinosa*, and southern bean mosaic virus on *Phaseolus vulgaris* L. are shown in Fig. 5 (5, 16, 17). The smooth curves fitting

the data in both figures are graphs of Equation 1. These data are in good agreement with the theoretical curve and are thereby consistent with the assumption that one virus particle is sufficient to cause infection if it happens to be favorably located. The curves which do not fit the data are graphs of Equation 2. Theoretical curves based on the Poisson series development for minimum required numbers greater than two would fit even more badly. There are dilution data for plant viruses in the literature which do not follow the course demanded by Equation 1 with the same high fidelity as the data here presented. As discussed in more detail in the theoretical section, Bald (7) has pointed out that these deviations are probably due to complicating factors such as changes in the state of aggregation with changes in concentration. The fit of these data to equations for 2, 3, or more particles is even worse than to Equation 1, for all of the errors involved tend to decrease the slope.

It would be desirable to have some objective means of deciding whether or not the data of Figs. 4 and 5 actually fit the graphs. The  $\chi^2$  criterion as usually applied is not suitable for this purpose.  $\chi^2$  is defined as the ratio of the observed variance to the theoretical variance. The usual formula given in text books was derived for the special case in which the theoretical variance is due solely to chance occurrence, as, for example, in the expected distribution of heads and tails when a coin is tossed. In the cases under consideration, the total theoretical variance is the sum of a term due to chance occurrence and one due to error of observation. This latter is true because, in fitting the theoretical dilution graphs to the data, it is necessary to assign a fixed value to  $N$ . This value is an average. It is certain that  $N$  varies from leaf to leaf, and that therefore the ratio of  $y$  to  $N$  is subject to an error. There is no way to estimate the variation of  $N$  from the data presented in the literature, and thus the goodness of the fit of the data of Figs. 4 and 5 cannot be evaluated by the  $\chi^2$  criterion.

*Systemic Infections and Related Considerations:* Bald (5) regrouped his data in such a manner as to consider a leaf or a half-leaf as a single susceptible unit. Any leaf with at least one lesion was considered infected. Data for several plant viruses, when treated in this manner, followed the graph of Equation 1. On first sight this approach apparently eliminates the ambiguity with respect to the total number of units inoculated. However, as was shown in the theoretical discussion, no real advantage ensues, for the probability of any leaf becoming infected is a function of  $N$ . A graph of the type of Equation 1 should fit the data only if the variation in  $N$  from leaf to leaf is small. Bald's data seem to represent a favorable case.

In general, this method of approach is inferior to that involving total lesion counts. Not only does it not obviate the uncertainty in the value of  $N$ , but also the resulting errors are not randomly distributed about the theoretical sigmoidal curve. This is true, because, in the region of almost complete susceptibility, it is not possible for the observed number of infected leaves to be greater than the number inoculated, but negative errors can occur due to occasional leaves having low values of  $N$ . Similarly in the region of great dilution there is the possibility of positive bias due to occasional leaves having abnormally high values of  $N$ . Hence, data treated in this manner should usually be expected to fit a graph with a slope somewhat less than that of Equation 1, depending upon the degree of variability in  $N$ . It must be emphasized that this concept of the influence of variation in host susceptibility upon the shape of virus dilution curves is far different from that based upon Gaddum's theory as advocated by Bryan and Beard (6). It does not conflict with, but rather supplements, the hypothesis that one virus particle is capable of causing an infection.

All cases in the field of plant virology wherein the host is inoculated by rubbing and the infection is judged by the development of systemic symptoms fall within the category under consideration. This is true also of the studies of Bryan and Beard (18) on rabbit papilloma virus. In their case a certain amount of virus suspension was spread over a definite area of scarified skin, and the occurrence of infection in the area as a whole was used as a criterion. The scarified areas will certainly vary in the number of susceptible regions they contain. Deviations of the dilution curve from the graph of Equation 1 should result, but the slope should always be less than that of the ideal curve. Furthermore, the length of time for obvious symptoms to develop should be a function of the virus concentration, for high concentrations of virus should be associated with high probability of every susceptible spot in a scarified area becoming infected and low concentrations should be associated with low probability. It is reasonable that a scarified area with many foci should develop gross symptoms sooner than one with few foci. The data of Bryan and Beard on rabbit papilloma virus are in complete agreement with these views. The dilution curves tend to deviate from the ideal in a negative sense and the time of development of obvious symptoms is a function of concentration.

In the consideration of Parker's data on vaccinia virus, it was ten-

tatively assumed that the inoculation of a fixed volume of virus suspension into rabbit skin was of the same class as injecting a suspension of bacteria into a perfect nutrient medium. The data obtained with the Board of Health strain tended to confirm the reasonableness of that assumption. However, results obtained with less virulent strains of vaccinia virus make it necessary to regard this assumption as being without general validity. Two obvious factors can contribute to departure from the ideal behavior in the case of vaccine viruses of low virulence. The first is the difficulty encountered in classifying inoculation sites, for strains of low virulence often cause extremely mild and indistinct lesions. The second is the possibility that only a small fraction of the virus particles injected find suitable environments for development. Thus, the injection might be analogous to scarifying the skin. The number of particles which do find suitable environments could depend upon accidental factors such as the amount of injury caused by the inoculation and the quality of the skin at the site. It could also depend upon the virulence of the strain of virus used. Considerations of this sort can account for the non-ideal behavior of strains of vaccinia virus of low virulence.

The results obtained by Parker (3) and by Parker, Bronson and Green (19) with various strains of vaccinia adapted to hosts other than the rabbit tend to bear out these views. The data for the *CV* strain of vaccinia, a strain adapted to chick embryos, and one extremely avirulent for rabbit skin, show deviations from the graph of Equation 1, as was pointed out by Bryan and Beard (6). Several other strains of low virulence gave similar results. In all cases except one, the deviations were such as to cause the average slope of the experimental curves to be somewhat less than that of the graph of Equation 1. Further, it was observed that the titer of the *CV* strain was many fold greater in chick embryos than in rabbit skin. In other strains better adapted to rabbit skin the reverse was true. These results can all be explained in the manner indicated above, a manner which takes into account the specific relationship between the virus and its host and also the contribution of the variation in host susceptibility, without in any way conflicting with the view that one virus particle is sufficient to cause an infection.

*The Applicability of the Theory of Gaddum:* Bryan and Beard (6) have contended that variation in host susceptibility is the factor of importance in determining the shape of virus dilution curves. They

have shown that their own data obtained with rabbit papilloma virus and the data of Parker on vaccinia virus can be made to fit graphs derived as indicated earlier from the hypothesis that the logarithm of the host susceptibility is distributed normally. On the other hand, we have shown that the same data can be understood in terms of the effect of dilution upon the chance occurrence of at least one particle in a susceptible region. The question that remains is: Which of these alternatives is more probably correct?

There are three lines of evidence which indicate that, at least in the

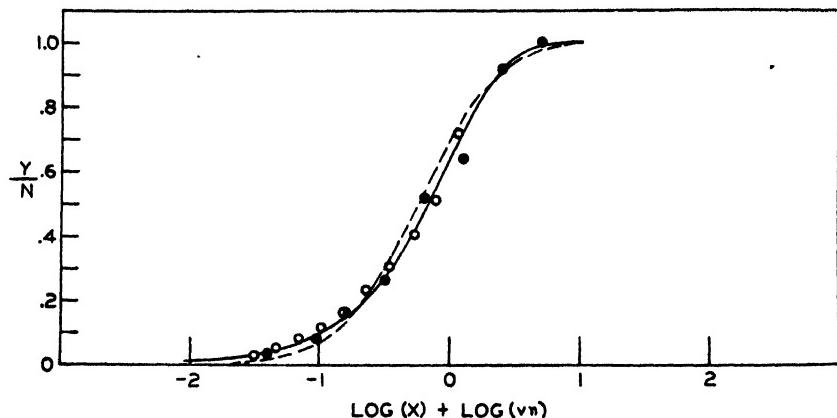


FIG. 6

Experimental Probability of Infection of Tobacco Mosaic Virus on *N. glutinosa*  
Plotted as a Function of  $\log_{10}$  of Virus Concentration

○—Data of Beale reported by Youden, Beale and Guthrie. ●—Data of Bald.  
Solid line identical with curve 1 of Fig. 1. Broken line identical with the broken line  
curve of Fig. 2.

case of certain plant viruses, the theory based solely on variation in host susceptibility is untenable. The first is that, in the case of tobacco mosaic virus on *N. glutinosa* leaves, the data obtained are sufficiently accurate to show that they fit the graph of Equation 1 better than the curve approaching it most closely which can be derived from the Gaddum theory. This can be seen in Fig. 6.

The second, an observation of more general significance, is that the dilution curves of all viruses tend to have the same slope. From Figs. 3, 4 and 5 it can be seen that dilution curves of three different strains of tobacco mosaic virus and potato X virus on *N. glutinosa*, those of

tobacco ringspot virus and cucumber mosaic virus on *V. sinensis* and southern bean mosaic virus on *P. vulgaris*, and those of certain strains of vaccinia virus on rabbit skin follow closely the same general graph. In addition, data obtained with other strains of vaccinia virus and with rabbit papilloma virus on rabbit skin deviate only slightly, and the deviations are such as to make the slope of the dilution curve less than that of the graph of Equation 1. The Poisson theory for one particle demands that all virus dilution curves approach the same ideal shape, regardless of the host or the virus used, subject only to general limitations of the sort described previously. The Gaddum theory does not require this. If the Gaddum theory is to be applied, it is necessary to conclude that the variability in host response to viruses is a constant running throughout wide segments of the plant and animal kingdoms. It is unlikely that such a complex restriction upon living things exists in nature. Therefore, it is unlikely that it is correct to apply the Gaddum theory to the interpretation of the dilution curves of any of the viruses considered here.

The third line of evidence is approached through the study of the infections resulting from mixed inocula. It is an essential feature of the equations based on the Poisson series that, while an infection can be caused by a single unit, a certain fraction, dependent upon the virus concentration, will be initiated by two particles, another fraction by three, and so on. The same is true with respect to the Gaddum theory, but the numbers will usually be quite different from those for the Poisson distribution. Evidence bearing on the issue can be gained by comparing theoretical distributions with distributions of mixed and unmixed infections obtained from experiments with mixed inocula.

In 1934 Kunkel (20) observed that, from single necrotic lesions produced in *N. langendorffii* Weinm. plants inoculated with a mixture of equal parts of aucuba mosaic virus and tobacco mosaic virus, usually one or the other virus could be reisolated, seldom both. Kunkel's inoculations were made by needle puncture in such a manner that lesions developed around only 2 to 5% of the punctures. The fact that most such lesions contained only one type of virus is quite in accord with the theory that infection follows the Poisson distribution for one particle. Since Kunkel's tests were confined to the region where only 2 to 5% infections were obtained, the writers undertook an experiment to determine the numbers of mixed infections obtained at "different virus dilutions.

Purified preparations of tobacco mosaic and aucuba mosaic viruses, containing 21 mg./cc. and 15.3 mg./cc., respectively, were obtained by

high speed centrifugation of juices from three weeks infected Turkish tobacco plants. Mixtures of the two viruses in the proportion to give equal numbers of lesions on *N. langsdorffii* were prepared and made up in serial dilutions with phosphate buffer at pH 7. The dilutions were applied in random order to sets of plants of *N. langsdorffii*, the number of plants varying with the dilution. The numbers of lesions produced per plant were found to be described approximately by the equation,  $y = 1350 (1 - e^{-74200z})$ , where  $z$  is the total virus concentration in g./cc. Lesions on *N. langsdorffii* produced by the  $10^{-2.5}$ ,  $10^{-4.5}$  and  $10^{-6.5}$  dilutions of the mixed inoculum were cut out, macerated, and subinoculated to *N. sylvestris* plants. The results obtained are given in Table I.

TABLE I  
*Distribution of Mixed Infections*

Log <sub>10</sub> concen- tration g./cc.	Distribution of virus recovered			Not infected	Total	Per cent mixtures				
	TMV alone	AMV alone	TMV + AMV			Observed	Expected			
							Poisson theory	Gaddum theory*		
-2.5	58	9	32	1	100	32	100	100		
-4.5	58	27	12	3	100	12	53.9	100		
-6.5	98	101	3	6	208	1.5	0.84	58.5		

\* Calculated on the assumptions that the dose of 50% response is  $10^{-6}$  g./cc. and that 1000 particles come into contact with a susceptible area at that concentration.

The percentage of mixed infections expected can be computed, first, from the Poisson series and, second, from the adaptation of the Gaddum theory. The computation for the first case involves only the calculation, by means of the various terms in the Poisson series, and the constants used to fit these data to Equation 1, of the percentages of lesions obtained in which two, three, four, etc., particles should have been present initially. From these figures, the numbers of compound infections expected can be evaluated by a simple application of the laws of chance, provided only that either virus is able to multiply freely in the presence of the other. The results are listed in Table I. The computation for the second case is not as unambiguous. It is necessary to assume that for a given plant the susceptibility is

about the same for both viruses. As was shown previously, the standard deviation of the distribution of the logarithm of susceptibilities must be about 0.5 logarithm. In order to have this theory apply at the lower concentrations, it is necessary to postulate that the average susceptibility corresponds to a dose containing a very considerable number of infectious units. As nearly as can be estimated, several million virus particles are applied to a leaf for each lesion produced at the concentration corresponding to the average susceptibility. The problem remains to evaluate the fraction of lesions in which both aucuba and tobacco mosaic virus particles should be expected to remain after the defenses have been exhausted. It can be shown that if a million particles can be resisted by an average cell, practically all lesions at all dilutions studied should be mixed infections. To give this theory the benefit of every reasonable doubt, a safety factor of one thousandfold was introduced, and it was assumed that the dose corresponding to 50% response, or the average dose, contained only 1000 particles. By utilizing the distribution of susceptibilities postulated, it was possible to compute the fraction of the lesions at each dilution which should initially have had more than one infectious particle after exhaustion of the defenses, and then from the laws of chance, the fraction of mixed infections was estimated. The results are listed in Table I.

It is obvious that the results of the experiment can not be rationalized to the Gaddum theory even when the assumptions made in obtaining the theoretical values were weighted a thousandfold in favor of the theory. The agreement of the observed values with the theoretical ones computed from the application of the Poisson series is not very good, but it is very much better than the above. The proportion of mixed infections observed is in good agreement with the Poisson theory only in the case of the  $10^{-6.5}$  g./cc. inoculum. On the other hand, it is clear that the proportion does increase when the concentration of the inoculum increases, as required by theory. The failure to obtain as high a proportion of mixed infections as expected at the higher concentrations is probably due to the difficulty of isolating the viruses from necrotic lesions. This difficulty is evidenced by the failure to isolate any virus whatsoever from 10 of the 308 lesions tested. Moreover, in a lesion initiated by both kinds of virus, the kind best suited to the host or that which grows more rapidly might crowd out the other. In the above data there is a strong indi-

cation that this may have taken place. At the lowest concentration, where the proportion of mixed infections is small, the lesions showing aucuba mosaic virus were equally numerous with those showing tobacco mosaic virus. This result should have been obtained, for the virus inocula were mixed in proportion calculated to give equal numbers of lesions. But at the higher concentrations, tobacco mosaic virus was recovered much more often than aucuba mosaic virus. On the assumptions that the two should have been equally numerous and that aucuba virus never crowds out tobacco mosaic virus in a mixed lesion, one can calculate from the data in Table I that 81, 43 and 1.5% of mixed infections were obtained where 100, 53.9 and 0.84% were expected. This result would be in satisfactory agreement with theory.

#### SUMMARY AND CONCLUSIONS

The development of an equation involving the Poisson series, applicable to the study of virus infectivity at various concentrations, was reviewed. The fundamental assumption underlying this development is that the occurrence of virus infection is dependent upon the chance occurrence of the required minimum number of virus units in the dose actually coming into contact with a susceptible region of the host. It was shown that the resulting equations can be applied without objection only in the case of virus host systems in which a primary lesion of unmistakable character is formed at the site of inoculation, and then only when the individual primary lesion is considered as the criterion of infection. When the method of inoculation is such that many foci of infection must be judged by gross symptoms, the results might deviate from the equations because of possible variability in the number of foci per entity showing gross symptoms.

The development of an alternative hypothesis was also reviewed. According to this view, the distribution of the number of positive responses to varying dose levels is due solely to the variability of host susceptibility. This theory is analogous to one used to account for the response of organisms to various drugs and other agents. It was shown that under special circumstances, this theory could lead to a final result resembling fairly closely that obtained for the theory based upon chance occurrence of the minimum requisite number of units in the dose.

A large body of virus infectivity data drawn from the literature was examined. It was found that all of the data examined tended to be

consistent with the theory that the quantitative response at various levels of dosage was due primarily to the chance occurrence of at least one infectious unit in the dose. The data excluded entirely the possibility that the response at various levels is due to the chance occurrence of a minimum of two or more units. Numerous cases of relatively bad fit of data to the theory were found, but in each instance, the discrepancies could be attributed to secondary factors such as variability in the number of susceptible regions within the site of inoculation, ambiguity in the criterion of infection and aggregation of the infectious unit.

The data studied also appear to fit the alternative hypothesis when suitable restrictions are applied. However, independent evidence was obtained to show that this theory is untenable. First, it was pointed out that some of the data obtained with tobacco mosaic virus can be seen to fit the graph in the equation based on the first theory better than the graph corresponding to the alternative hypothesis. Second, it was pointed out that if the alternative hypothesis is to be accepted, it must be concluded that the variability in the response of all virus-host systems, both plant and animal, must be essentially the same. It is unlikely that such an arbitrary restriction has its counterpart in nature. Third, experiments were carried out which showed that, when a suitable host was inoculated by a mixture of two strains of tobacco mosaic virus to produce local lesions, from which the one strain, the second strain, or both strains could be recovered, the number of compound infections varied with the concentration of inoculum in a manner more nearly in agreement with calculations based on the theory involving the Poisson series than with those based on the alternative hypothesis.

These results lead to the conclusion that the only tenable theory at present available for explaining the character of the quantitative response of hosts to various doses of viruses is that the probability of infection is related to the probability of finding the minimum requisite number of infectious units in an element of volume which comes into intimate contact with a susceptible focus in the host. If this theory is correct, the data prove conclusively that for the viruses thus far studied, one infectious unit is sufficient to initiate an infection. On the basis of present knowledge, therefore, the proposition that one infectious unit can cause virus infection is the most reasonable starting point for speculation concerning the nature of virus reproduction.

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## Book Reviews

**Allgemeine und Milchwirtschaftliche Mikrobiologie.** By WILLY DORNER, Chief of the Bacteriology Department of the Swiss Dairy Research Institute, Liebefeld-Bern. Huber & Co., Frauenfeld und Leipzig, 1943. 211 pp. + 16 plates of illustrations.

The book is a textbook of dairy bacteriology, with an introduction into bacteriology generally, but the emphasis throughout is on application to the dairy industry. Of the 200 pages, 72 are devoted to general bacteriology, 90 to a discussion of the importance of micro-organisms in dairy industry, and the remaining 37 pages are laboratory directions for general and dairy bacteriology. The book is obviously intended primarily for dairy schools, comparable in this country to short courses offered by the various state colleges of agriculture. A thorough knowledge of chemistry is not taken for granted, and chemical formulas are mentioned only in footnotes or small type.

The book is remarkably well written, in short and clear sentences, with a great economy of words. It is surprising how much thorough knowledge the author has been able to concentrate in the small space allotted to scientific discussion. He even managed to include the Meyerhof-Embden scheme of lactic fermentation, although in small print. The author is well informed in modern bacteriology, and his discussion is up-to-date. He adopted the system of the latest edition of Bergey's Manual as the best system of bacterial taxonomy.

About one-fifth of the entire book is devoted to a discussion of Swiss cheese, and this discussion is so expert and thorough that students and manufacturers of Swiss cheese may well profit by reading these chapters.

The book is illustrated by 50 good microphotographs of bacteria, and by photographs of colony types.

OTTO RAHN, Ithaca, N. Y.

**The Bacterial Cell in its Relation to Problems of Virulence, Immunity, and Chemotherapy.** By RENE J. DUBOS, George Fabyan Professor of Comparative Pathology and Professor of Tropical Medicine, Harvard University; with an addendum on "Nuclear apparatus and cell structure of rod-shaped bacteria" by C. F. ROBINOW of the Strangeways Research Laboratory, Cambridge, England. Harvard University Press, Cambridge, Mass., 1945. xix + 460 pp. Price \$5.00.

The basic idea of this excellent book is that a comprehensive knowledge of the chemical composition, biochemical behavior and structural organization of the cells of pathogenic bacteria will inevitably lead to the development of rational and more effective methods of combating bacterial diseases. This thesis is very ably presented and supported by abundant evidence obtained from many sources. The first four chapters present a picture of the organization of the bacterial cell as revealed by

microscopic examination, staining reactions, physicochemical behavior, and enzymatic and immunological analysis. The fifth chapter deals with various aspects of variability in bacteria, both morphological and biochemical. The following three chapters show how knowledge of the structure of the bacterial cell can be applied to the interpretation and further investigation of infectious processes, particularly in relation to virulence, immunization and chemotherapy. The final chapter on "Trends and Perspectives" begins by emphasizing the transition of bacteriology from a strictly empirical to a more theoretical science and ends with a discussion of the importance of specificity and variability as fundamental biological properties of bacteria.

The great merit of the book is that it unifies and clarifies many at first sight apparently unrelated facts concerning the biochemical architecture of the bacterial cell and its reactions in infectious processes. The reader cannot help but be impressed by Dr. Dubos' breadth of view and by the clarity of expression which makes even relatively complex immunological phenomena easily intelligible to the non-specialist. The book also stimulates thought to an extraordinary degree; almost every page presents or suggests some further line of investigation.

There are a number of minor defects. The text contains more than a few typographical errors. Several of the graphs are poorly drawn or inadequately labeled, for example, Fig. 12, p. 86 and Fig. 18, p. 139. A few omissions or errors of fact were noted. For example, on page 39, line 3, it is stated that "it is not known whether the aerobic sporulating bacilli can . . . produce capsular material of polysaccharide nature. . . ." Actually, a number of aerobic spore-formers have long been known (Beijerinck) to form large quantities of a capsular levan from sucrose. On p. 138-140 it is stated that the mean cell volume of all bacterial species with the possible exception of the diphtheria bacillus, decreases in size during and after the logarithmic growth phase. On the contrary, several bacteria, such as *Acetobacter* and various soil mycobacteria and corynebacteria, have been observed to increase in size with age.

This outstanding book should be of great interest to all microbiologists as well as to many investigators and students in related branches of science. It could well be made required reading for second year students of microbiology.

H. A. BARKER, Berkeley, Calif.

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## ERRATUM

Page 60, sixth line from bottom, the value 0.5 Å.U. is incorrect. It should read 4.5 Å.U.

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